

Public health and public conscience

A rash of anxiety in Britain about prion diseases apparently caused by infectious proteins suggests it is time for the government to change its line on the risks to people of bovine spongiform encephalitis.

"So tell me, does eating beef from cattle infected with BSE expose me to the risk of going down with Creutzfeldt-Jakob disease, otherwise CJD?" That question aptly expresses one public anxiety in Britain since 1986, when BSE (bovine spongiform encephalitis) was first recognized to be the cow's equivalent of scrapie in sheep, and to be prevalent among British dairy herds. The question is a reasonable one if asked by, say, a patient of a physician, or by a dinner-table acquaintance of a biomedical researcher. The underlying anxiety is also understandable; if the scrapie of sheep can be transferred to the BSE of cattle, why should not BSE become CJD? Moreover, the rejoinder that "There is no evidence that this happens" is unconvincing. The temptation is to reply, "So far". So how should the research community respond?

In Britain, this is hardly the most propitious time to answer such a question. Last week, the Department of Health opened a help-line to deal with enquiries from alarmed women who may have been given gonadotrophin extracted from the pituitary glands of dead people (see page 98); now there is evidence that CJD can be acquired in that way. A more formal search is already under way for those treated in their youth with growth hormone from the same potentially contaminated sources, as well as those suffering operations on the skull and its interior that may have been repaired with contaminated human tissue. On the heels of a scandal at a hospital in Birmingham in which it emerged that nearly 20 per cent of a sample of 200 patients had been wrongly diagnosed (negatively and positively) as having bone cancer (the diagnoses of more than 1,000 other cases are still to be checked), the public mood is out of sympathy with the biomedical profession.

The case of Professor Jean-Pierre Allain, recently sent to jail by the French courts, is relevant (see *Nature* 364, 267; 1993). Allain (who is appealing against the verdict) was convicted for not having warned French haemophiliacs that they were being given clotting agents probably infected with HIV, his protests within the National Blood Transfusion Centre notwithstanding. The justice of that verdict is questionable. Means of testing for HIV had only recently become available, while neither Allain nor his fellow-defendants were those chiefly responsible for deciding to continue distributing contaminated factor VIII "while stocks last". But where would those responsible in Britain for past and present decisions on prion diseases stand if they found themselves before the same French court?

The experimental transfer of scrapie to mice dates back to

1961, but it may be unfair to suppose that the mouse was then a model for CJD. But long before the transfer of scrapie to Syrian golden hamsters (in 1981), the analogy between scrapie and CJD was commonly written about. Should physicians then have abandoned the use in therapy of material from dead people on the grounds that there would be a risk, perhaps very small, of transferring CJD? Or should they, in the absence of alternatives, have warned the potential beneficiaries of the risk? In retrospect, it is easy to say that they should have followed one of these courses. As the French courts see it, not to have done so would have qualified them for jail.

The question of whether BSE may cause CJD in those who eat infected beef is different. Certainly there is no known case of cross-infection (but BSE has not been around for long enough to test the long induction period). Nor is there anything like an understanding of the mechanism of any of these diseases. (The infectious agents appear to be protein molecules called prions; nobody knows how they might replicate in cells, and there is no unambiguous distinction between infectious prions and the proteins produced naturally in neurons and some other cells.) On the other hand, there are examples in the literature of the transfer of scrapie-like diseases between species. Will some judge in future say that, in the middle of 1993, it must have seemed probable that what happens between sheep and cattle might also occur between cattle and people?

To raise that awkward question does not imply that the British are negligent in the precautions they have taken to prevent the consumption of contaminated beef. The raw materials of cattle feed are now tightly controlled, while there are rigorous inspections of meat sent for sale. But the length of the induction period in cattle must mean that some infected beasts are missed. The chances are that the precautions have substantially reduced the risks. The chance that the risk has been reduced to zero is very small.

Physicians should prudently tell patients just that. Biomedical researchers, less directly responsible, have a different duty; to press for a more urgent concentration of resources on what is, in any case, an intriguing field. If, as some suggest, the difference between a normal and an infective prion is merely the molecular conformation, that would be a prize of understanding well worth winning. The real dilemma lies with those who advise the government, and who are no doubt made painfully aware of the economic importance of the beef industry. Soon they will have to stop saying there is no evidence.

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Referees' pain ahead

The Soros fund will need the help of an army of referees in the weeks ahead. It should be given what it needs.

THOSE who referee articles intended for publication or research grant applications for public and private agencies had better brace themselves for another shock — the flood of grant applications from researchers in Russia and the other republics of the ex-Soviet Union from the organization set up by Mr George Soros to spend his \$100 million (see *Nature* 364, 749; 1993). The deadline for applications is 25 September. Applicants for grants, for sums up to \$100,000, have been asked to nominate their own referees. (In a delicate touch, the organizers have asked them to vouch for their referees by citing at least one publication in a respectable journal for each referee, which in itself will ensure that a high proportion of the referees are in the West.) Each application may be sent to up to six referees, and there may be 5,000 or so applications.... Plainly the world's referees are in for some hard-working weeks.

It is to be hoped that they will rise to the challenge. The Soros fund is a unique and imaginative contribution to the survival of ex-Soviet science and, in that role, deserves the support of all who are able to help. The first part of the project, to award grants of \$500 to people able to boast of three papers in internationally recognized journals, seems to have confounded the scepticism of those who complained of the apparently mechanical method of selection. (The distribution of cheques in Moscow is now complete; this week they will be going out to Novosibirsk and Kiev.) That money, and the \$10 million so far spent on travel, has helped enormously to keep ex-Soviet scientists in touch with the rest of the world. But the major research grants now in prospect, on which the advice of referees is needed, will cut deeper into the ice.

Will there be enough good applications? A year ago, when this idea was hatched, it seemed likely that the Soros fund would be overwhelmed. But now there is less certainty on that score. So many able people have since left, perhaps only for the time being. But that in itself will be something worth knowing, while opportunities will certainly abound for making grants to groups well placed for the recruitment of students. Whatever the case, the grant scheme now coming to fruition will have left two permanent lessons; first, it will have demonstrated that a system of awards based only on merit is feasible even in the former Soviet Union. Second, it will have provided ex-Soviet science (not to mention many Western foundations) with a proof that even the most complicated tasks can be done quickly and well.

There remains the question of what will come after the Soros fund. The plan is to award between 500 and 2,500 grants between now and the end of the year, when the \$100 million will almost all have been spent. Thereafter, there will remain for a year an organization that is part travel agent and part procurement agency and that is skilled at moving money about and talking to the banks. It will be a shame if that is

allowed to turn into dust, especially when the West is full of foundations that say they would do anything they could to help save ex-Soviet science if only they had a mechanism for doing so. What ex-Soviet science (and the rest of us) need is that the Soros mechanism should be made permanent and used for a diversity of funds. Who will take up that challenge? □

Reinventing government

The United States government would do well to follow the advice of Gore's task force.

GOVERNMENTS are forever studying their own administration under the rubric of reform. Sometimes they even follow the recommendations of the task forces they create for the purpose. The United States seems next in line. Vice President Albert Gore, since his inauguration last January, has been the head of what has been called the National Performance Review, intended to examine and, as necessary, streamline the immense bureaucracy of the United States. Gore's report, out this week, will no doubt seem to many familiar vested interests as a signal to begin another bout of lobbying. This time, the research community should not be left behind, on at least two important issues.

First, and perhaps most politically controversial, Gore recommends that Congress should allocate resources to federal agencies on a budget cycle that spans two years rather than one. In principle, were this to be accepted (which is doubtful), it would go some way to meet the research community's plea for more stable funding. Simply knowing how much money is available, some argue, would contribute to progress in research by relieving the psychological stress of uncertainty. This year's budget cycle, which has thrown several important projects into limbo, shows how damaging can be the hesitations of the Congress even for projects that are eventually supported. Whether a two-year rule would mean less time spent writing grant proposals is less clear.

Gore has also intelligently suggested abandoning the government's monstrous hiring system (the Federal Personnel Manual's rules and regulations run to 10,000 pages), which often discourages able scientists from taking positions in US science agencies such as the National Institutes of Health and the National Science Foundation, at which excellence in research is greatly needed. Gore would hand over to the agencies themselves the responsibility for recruiting employees (and offering bonuses and other incentives) to what is known as the Senior Executive Service. It is a suggestion that should be implemented — soon.

But that may not be all. Gore's report, called *Reinventing Government*, is likely to have implications throughout the US government service. The trouble is that they will be signalled only in the fine print. Who will read that with an eye for the implications it may have for the research enterprise? There are many in the British research community still kicking themselves that they did not realize that a plan in 1986 to privatize government agencies would chiefly affect laboratories. □

CNRS under fire from staff

Paris. About 20 French researchers have formed an association, SOS Recherche, to fight against what they describe as abuse of power, favouritism, and unfair discrimination by the country's largest science agency, the Centre National de la Recherche Scientifique (CNRS).

Each of the association's members claims to have suffered as a result of such pressures. For example, the president of the new body — its title stands for "Scientists opposed to the sclerosis of research" — is Claude Reiss, a geneticist who has been in conflict with the CNRS for more than four years.

He claims that officials tried to close his laboratory at the Institut Jacques Monod (IJM) in Paris, where he works on the structure and dynamics of the genome, and to strip him of his job, for reasons that were personal rather than professional.

Following a dispute over a researcher who had asked to work in Reiss's laboratory, Claude Paoletti, then the CNRS's director of life sciences, froze all recruitment to the laboratory on the grounds that a national review board had said that it was not publishing enough scientific papers.

When Reiss showed that its publication rate was more than twice the CNRS average, Paoletti shifted his ground and ordered Reiss's laboratory to be closed because it lacked "real strategy."

Reiss took the CNRS to an industrial tribunal, and the organization backed down just before the case was to be heard. Reiss claims that this action confirmed his suspicions about the CNRS's motivation, although

Paoletti maintains that his decisions were professionally correct, and that there was no personal conflict with Reiss.

But Reiss is now once again facing closure of his laboratory. In June, Jacques Ricard, director of IJM, ordered Reiss to vacate part of his laboratory and join another group at IJM until a laboratory at Gif-sur-Yvette became free. "When I was appointed in January 1991, the situation between Reiss and Paoletti was deadlocked", says Ricard. "I thought it best that he [Reiss] should go elsewhere."

Ricard says the move was simply part of his plans to change the staffing at IJM. Reiss says he will go if his name is cleared, if he is allowed to recruit staff, and if the CNRS compensates him for legal costs and loss of research funds.

Pierre Tambourin, Paoletti's successor, says he is confident that a satisfactory arrangement will be quickly found. CNRS says that the incidence of such lawsuits is low considering that it employs more than 11,000 researchers. But resentment about its procedures remains strong.

Another researcher, Hasan Parves, for example, has found himself caught between two administrations. The CNRS is insisting that he move his neurotransmitter laboratory from Paris to the University of Reims, where his wife has recently been given a post. But the university says it does not want him, and will not provide him with either space or money.

The president of the university, Claude Savarin, has stated that the reason for its

opposition is that Hasan's wife was appointed "against the wishes of the university", after she defeated the university's preferred internal candidate in a national competition.

Now, even though Hasan has nowhere to go, the CNRS insists he must leave the laboratory he built up over 23 years at the University of Paris South "as soon as possible". Hasan himself is bitter. Despite having published more than 190 papers in international peer-reviewed journals and edited over 16 books, the CNRS has repeatedly denied him research funding, providing just FF25,000 in 23 years. Hasan has had to raise his research funds through collaborations with groups in the United States and Japan, as well as industrial contracts. And despite his success at doing so he has never climbed above the bottom rung of the CNRS promotion scale, and has been denied promotion for 15 years in succession.

The CNRS denies that it is excessively tough on its researchers. It says its only obligation is to pay them a salary, and that it has a duty to fix research priorities and to allocate resources within these priorities.

According to Reiss, however, there is a basic injustice in placing the responsibility on researchers to prove improper treatment by CNRS. To do so, they must go to an administrative tribunal. But this can take several years, by which time the researcher's career and professional reputation may have suffered irreparably.

Another problem targeted by SOS Recherche members is a lack of openness in the CNRS which, they say, encourages nepotism and abuse of power. CNRS officials and review committees are widely acknowledged to earmark recruits and promotions to people from their own or colleagues' laboratories (see *Nature* 352, 555; 1991).

Furthermore, French scientists are civil servants and can be dismissed for publicly criticizing government policy or the administration of research. Although such laws are rarely enforced, their existence engenders a reluctance to speak out against the scientific establishment.

The solution, suggests Reiss and other SOS members, is stricter adherence to proper disciplinary procedures, and faster and more open hearings of scientists' complaints. Both, they say, would be in the interests of French science.

Declan Butler

Physicists appeal against rough justice

Paris. For the past six years, Gilbert Reinisch, a physicist working on micro-electronics, has been contesting the way in which the CNRS stripped his research funding and forced him to leave Nice Observatory without any formal disciplinary procedure.

Reinisch had been at the observatory for 17 years when he and his colleague, Jean-Claude Fernandez, were forced out in 1987 by Raymond Michard, the then director. The official reason was that they had been working abroad when they should have been meeting a Soviet guest.

But Reinisch claims the real reason was that they had edited an internal newsletter criticizing aspects of the management of the observatory. Michard, who is now retired, last week refused to comment; but CNRS documents support Reinisch's hypothesis.

The researchers immediately filed a lawsuit against the CNRS, which later decided that they should be reinstated. Philippe Delache, the new director, subsequently took them back, but working next to astronomers rather than in their physics laboratory.

In December 1990, following a further dispute over computer files, the director ordered both researchers to leave the observatory within two weeks. The following month the CNRS sent Reinisch and Fernandez to share an unequipped room in a geology institute 20 km away.

Later in 1991, the administrative tribunal of Nice ruled that Michard's action had been an abuse of power and should be revoked. But the CNRS has never reinstated the researchers. The final chapter occurred in the early morning of 6 June last year. Removal men, supervised by the CNRS regional director, packed the researchers' archives at the observatory into 58 cardboard boxes and shipped them to their new institute. **D.B.**

Nature news staff

David Dickson has been appointed News Editor of *Nature*. Alison Abbott has become Senior European Correspondent, and will continue to be based in Munich. Declan Butler has joined the *Nature* staff as European Correspondent, and will be based in Paris.

Boson competition lifts prospects for LHC

Keele. William Waldegrave, Britain's minister for science, said last week that reading the successful entries in a competition to describe the significance of the Higgs boson had increased his enthusiasm for the construction of the Large Hadron Collider (LHC) being planned for the European Laboratory for Particle Physics (CERN) in Geneva specifically to search for the particle.

His expression of support comes at a time when Britain's particle physicists are preparing to tighten their belts even further in order to pay for their participation in the experimental programme on the LHC. Even so, they are warning that paying for the construction programme within current budget limits — and without, for example, participation by the United States — could lead to the suspension of all experimental work on other colliders for the five years before the LHC's planned opening in 2001.

Waldegrave was speaking during the annual meeting of the British Association for the Advancement of Science at a ceremony to award prizes for the competition which he had launched earlier this year. At a conference of the Institute of Physics, he had offered a bottle of vintage champagne — paid for, he emphasized, out of his own pocket — for the best explanation of the nature and significance of the Higgs boson written on a single side of A4 paper.

In all, 117 entries were received. And in the event, five winners were selected. Three are individual British physicists: Roger Cashmore of the University of Oxford (and chairman of the particle physics committee of the Science and Engineering Research Council), Tom Kibble of Imperial College London (widely credited for much of the theoretical argument for predicting, together with Peter Higgs, the existence of the Higgs boson); and David Miller, of University College London.

Each received a bottle of champagne as promised, as did the two other winners: a joint entry from Mary and Ian Butterworth of Imperial College, London, and Doris and Vigdor Teplitz, of the Southern Methodist University in Dallas, Texas; and Simon Hands, of CERN's theory division.

Miller's was the most imaginative answer. The others gave straightforward, if lucid, explanations of how the existence of the postulated Higgs particle would confirm the idea that particles gain mass by interacting with a field defined by certain parameters (the Higgs field). Miller chose to illustrate the idea by comparing the influence of such a field on individual particles to the clusters that would form around — and give momentum to — an "ex-prime minister" as she (*sic*) walked through a room of

party workers, and the Higgs particles to the clusters by which a rumour would pass through the same gathering.

Commenting on the results of the competition, Waldegrave said that the entries had not only demonstrated to him why the particle was important, but had also increased his sympathy for supporters of the LHC. "If we cannot find the money — and that is going to be hard pounding — I will recognize it as a loss" he said.

Waldegrave's remarks come at a time when European governments are preparing their responses to CERN's request to be given the go-ahead for the construction of the new accelerator, which would be built in the tunnel currently occupied by the Large Electron-Positron collider (LEP).

CERN has said that it can build the new machine without any net increase in contributions from member states. Estimated to cost £7 billion (considerably less than the \$11 billion for the rival Superconducting Super Collider already under construction in the United States), building work on the LHC would, if approved by the end of the year, be completed in 2001.

But meeting Britain's CERN subscription of £55 million a year while at the same time preparing for full participation in experiments on the LHC is already requiring some difficult decisions by the physics community. The SERC has now endorsed a financial strategy put forward by its particle physics committee, which would achieve these objectives only by cutting back severely on planned experiments both on LEP and on Germany's electron-proton collider (HERA) at the DESY laboratory in Hamburg.

The £16 million saved in this way over the next eight years, even when added to the £12 million cut in Britain's CERN subscription negotiated last December, will not, according to the SERC panel, be sufficient to meet the total reductions needed to free funds need for the LHC. Of various options suggested, the one considered by the panel to be the most likely to succeed would be to invite contributions to the LHC from countries (in particular the United States) that are not members of CERN but make use of its facilities.

As far as Britain is concerned, says the panel, if a further reduction in its CERN contribution is not achieved, the only option will be to withdraw completely from research projects at either LEP or HERA (and perhaps even both) in 1995 or 1996. This move could leave physicists with virtually no experimental programme for the five years preceding the opening of the LHC.

David Dickson

Stay-hard tomato carries its label with pride

Washington. Chicago has become the first American city to pass a local law requiring that all food produced by genetic engineering must carry a label indicating this fact — a worrying prospect for biotechnology companies hoping that research on plant genes will lead to the more efficient production of a swath of foodstuffs.

Chicago's move reflects a growing consumer opposition to genetically engineered The Pure Food Campaign, led by lobbyist Jeremy Rifkin, describes the Chicago law as "a devastating blow to the biotech industry", and has called on other localities to follow suit. The Campaign says that genetically engineered foods carry potential health risks, and that polls show most Americans to consider the transfer of genes from one species to another is "unethical".

New York already has a similar law in the pipeline. An attempt will be made to enact it "if the Food and Drug Administration [FDA] doesn't act soon", says a spokeswoman for the city consumer affairs department.

But the FDA itself does not appear to see any need to rush. It has just finished taking comments from interested parties on its existing policy, which requires labelling on a genetically engineered food only if the treatment has had a significant effect on the consequences of use.

And not everyone is worried about the prospect of food carrying a "genetically engineered" label. Calgene of Davis, California, whose plan to start selling its "flavour saver" tomato this autumn has caused much of the fuss, intends to put such a label on the tomato with pride, regardless of the law. The flavour saver has been produced by disabling a gene which contributes to the softening of tomatoes. It will be sold at a premium, and labelled clearly. Stephen Benoit, a spokesman for the company, says that "people have been promised a better tomato so many times before, they want to know why they should believe the promise this time".

"Calling a tomato genetically engineered tells you nothing about it at all", says Dr Eric Flamm of the FDA, who says the agency will analyse and assess the comments received in the usual way. "The Calgene tomato will be labelled at the choice of the company. There are no other products on the horizon, so I don't see any time crisis."

But the Biotechnology Industry Organization (BIO) is taking seriously the threat of adverse reaction from consumers. It complains that the Chicago law was passed by council members with little consultation, and plans to work with lobby groups from the food industry to have the law repealed before it is due to take effect on 1 October.

Colin Macilwain

RIKEN wins reviewers' praise

Tokyo. The work of Japan's Institute of Physical and Chemical Research (RIKEN) near Tokyo, the country's leading basic research organization, has received high marks from a team of scientists from Japan, Europe and the United States who have carried out an external review of the institute.

In a report to be released today (9 September), the reviewers praise the general level of research being carried out at RIKEN, the standard of its facilities, and the procedures that have been introduced to check that research quality is being maintained.

However, as with a similar review of the physics department of Tokyo University carried out by foreign examiners in January, the reviewers say that the institute should open up its employment practices to attract more of the world's most talented scientists (see *Nature*, **362**, 387; 1993).

In contrast to Europe and the United States, where visiting committees are widely used to assess the quality of research in universities and other institutes, such committees are a new phenomenon in Japan. But they are rapidly gaining popularity as the scientific community seeks to increase its output of basic research.

RIKEN's team of 15 external reviewers, which visited the institute in June, was headed by Heinz Staab, director of the Max-Planck Institute for Medical Research and included George Clark of Massachusetts Institute of Technology, and Nobel prizewinner Heinrich Rohrer of IBM Zurich.

In a report on their three-day

visit, the reviewers describe RIKEN's research and facilities as "excellent". Particular praise is directed at the institute's Frontier Research Programme, which employs many foreign scientists — including several project leaders — and which is described as "fostering innovative research of exceptionally high quality and importance".

The reviewers also applaud RIKEN for the way it assesses and monitors the quality of research performed by its scientists. Since 1983, RIKEN has carried out regular external reviews of its chief scientists and their laboratories, an unusual practice in Japan where research leaders tend to protect their projects jealously from outside monitoring.

The reviewers point out, however, that there are almost no foreign scientists on the permanent staff of RIKEN. Those engaged in the Frontier Research Programme are employed on contracts, and there is only one

foreign senior scientist among the institute's 313 permanent research staff.

The review team calls for all jobs to be widely advertised. It also suggests extensive use of external referees, including non-Japanese, to assess the international reputation of senior scientists applying for appointments at the institute.

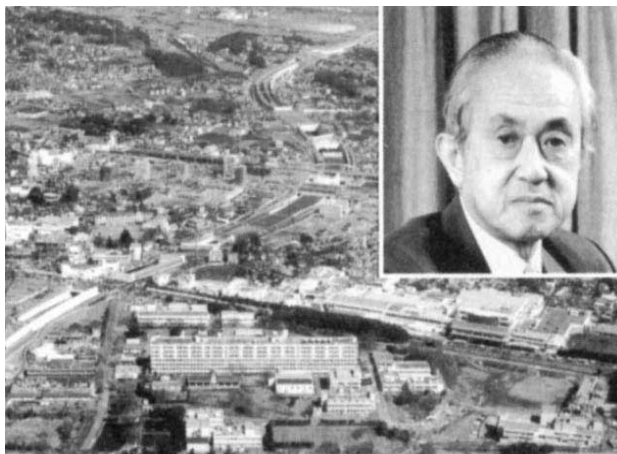
Criticism is also directed at RIKEN's policy of placing an upper age limit of 32 on those appointed to permanent positions below chief scientist. The institute justifies this policy as being necessary to maintain the youth of its research staff. But the reviewers feel that the age limit is too low, as it excludes many talented postdoctoral scientists, and that it should be raised.

In addition to the main report being made public this week, the review team has also delivered draft reports prepared by five subcommittees on research in individual laboratories to Minoru Oda, the president of the institute. These reports will be kept confidential; according to Oda, some of the laboratories are considered to be "surprisingly good", but others are described as being "out of date".

But Oda, who is due to retire shortly, is happy with this conclusion. He believes that, if at least one third of an institute is doing good research, "the rest will follow"; and he says he is pleased that the review shows that this criterion is easily being met at RIKEN.

The institute's board of executive directors is to set up a committee later this month to respond to the review team's recommendations. Unlike Tokyo University, which has made no decision on the future of the review process RIKEN is to repeat the exercise every two years.

David Swinbanks



RIKEN's president Oda (Inset) and labs

Japan to boost technology research

Tokyo. Japan's Ministry of International Trade and Industry (MITI) has asked the government to increase its funding for the Intelligent Manufacturing System (IMS) project — an international programme launched in 1990 and aimed at developing the automated factories of the future — by 48 per cent next year, to a total of ¥1.6 billion (\$150 million).

An even bigger increase is being requested in the amount of money MITI would like to spend on the Real World Computing project, a follow-up to the fifth generation computer project. The ministry wants this to grow by 59 per cent to ¥5.7 billion; and it also hopes to provide a further ¥1.4 billion for the final year of the fifth-generation project.

Meanwhile, the Science and Technology Agency (STA) is hoping to raise the budget for its Exploratory Research for Advanced Technology (ERATO) programme by 8.7

per cent, to permit the launch of four new projects. STA also wants to increase Japan's contribution to the International Thermonuclear Experimental Reactor (ITER) by 22.5 per cent, to ¥8.1 billion.

Both MITI and STA have submitted ambitious budget requests for research and development next fiscal year, which starts on 1 April, and have managed to avoid the worst of the squeeze on public spending being imposed by the Ministry of Finance (see *Nature* **365**, 7; 1993).

The requests will be trimmed back by the Ministry of Finance before approval by the cabinet at the end of this year. But, on the basis of previous experience, any changes are likely to be relatively small.

MITI's budget request, for ¥301 billion, is 7.5 per cent higher than this year. One prominent feature is a request for a 16.5 per cent increase in funding for computer networks between MITI's institutes, to ¥2.1

billion. This coincides with STA's bid for ¥1.1 billion to establish a high-capacity "backbone" computer network linking national research institutes and universities.

STA's request is 6.1 per cent up on this year at ¥617 billion. This includes an 8.9 per cent rise, to ¥158 billion, in the agency's space budget. Part of this money will be used to cover the rising costs of Japan's contribution to the US Space Station.

No increase is being requested in Japan's contribution to the Human Frontier Science Programme, based in Strasbourg, France. This is provided jointly by MITI and the STA, and the total requested is ¥4.2 billion, the same as last year. But the recent rapid appreciation of the yen means that, when calculated in dollars, the budget for the international programme may well rise above this year.

David Swinbanks

Prion disease scare

London. For the past ten days, Britain has been plagued by anxiety over prion disease largely because cases of Creutzfeldt-Jakob disease (CJD) have been linked to the use of pituitary and other material taken from cadavers. The anxiety has sprung from two separate causes. The more predictable has been the use of hormones extracted from cadaver pituitaries between 1956 and 1985, since when synthetic material has been used.

More surprising is the practice in neurosurgery of using a lyophilized preparation of outer brain-case membranes taken from cadavers as packing in the skull after brain surgery. The material used, trade-marked Lyodura, is manufactured by Braun Melsungen AG in Germany, but has not been used in Britain since its licence was withdrawn in 1991.

The latest anxiety has also been strengthened by fears that bovine spongiform encephalitis (BSE), still prevalent among British cattle, may yet appear as Creutzfeldt-Jakob disease in people.

Of the hormones extracted from pituitaries, the use of human growth hormone is the longest established. A project at the Institute of Child Health in London has been commissioned to trace the estimated 2,000 adults who were treated with the hormone as children or young adults.

The more recent use of pituitary gonadotrophin as a treatment for female infertility has thrown up more immediate problems for the British Department of Health, notably with publicity for four cases in Australia of women developing CJD after treatment with pituitary hormone.

In the early days, the extraction of hormones seems to have been the responsibility of the Medical Research Council, and was carried out at a Cambridge laboratory. Because pituitary extracts were pooled, neither batch numbers nor other identifiers survive to guide the search for people who may have been infected.

Meanwhile there is little to report on the BSE front. The rate of infection among cattle has not begun to fall, as the Southwood report in 1986 predicted it would have done by now. A committee under Dr David Tyrrell is now monitoring developments. But an interim report last year found no basis for changing the present policy of seeking to restrict the infection of cattle by the rigorous avoidance of sheep offal in cattle feed.

Although it is accepted that CJD and BSE are essentially similar, and that both are prion diseases (as is scrapie in sheep), there has not been unambiguous evidence of trans-specific infection of a person with CJD. On the other hand, British policy on cattle ex-

plicitly acknowledges that transfer can occur between sheep and cattle.

The position seems to be what it has been for some months. The protein part of the prion entity is known to be a normal part of the human genome (on chromosome 20), which is inserted in the outer membrane of neurons and certain other cells. Its function is unknown. A strain of mice bred to lack the gene was found last year (*Nature* 356, 577; 1992) to develop apparently normally, at least for seven months.

The course of prion disease is accompanied by the accumulation of an aberrant form of the protein within vesicles in the cell. In what way these molecules differ from the protein that remains anchored to the outer membrane is not yet known, nor is there any clear indication whether the normal or abnormal prion protein may be loosely associated with other entities, perhaps nucleic acid polymers.

The sheep, mouse and human prion proteins are all very similar, but the process of logging mutations of the human gene is no doubt far from complete. One intriguing feature of the known mutations of the human prion protein gene is that some are marked by the appearance of up to 9 eight-codon (24 nucleotide) repeats in a region near the front end of the gene already replete with them. But there is as yet no clear link between the structure of the gene and either the mechanism or susceptibility to disease.

John Maddox

More graduates — but fewer jobs

London. Britain's universities are producing more science and engineering graduates than ever before, according to figures released last week by the Universities Statistical Record. But at the same time the number of such graduates finding jobs in industry has been falling sharply, suggesting that the lack of trained scientists in the work-force remains, at least for the present, a problem of demand rather than supply.

The latest figures show that, contrary to widespread impressions in the media, the number of UK-based students graduating in science subjects rose by 7.6 per cent between 1991 and 1992, following a 3.0 per cent decrease the previous year. This news should temper much of the gloom caused two weeks previously by news of a continued decline in the proportion of school students taking science subjects at A level.

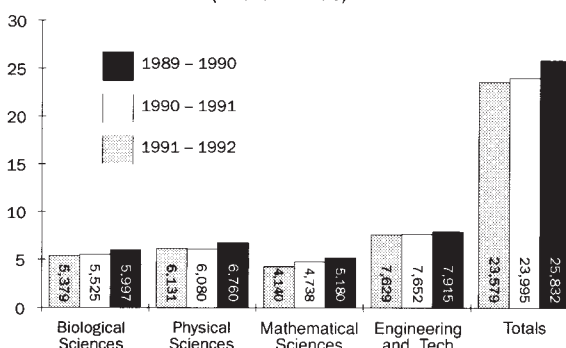
The trends for the physical science are typical. The number taking A-level physics dropped by about 10 per cent between 1992 and 1993. But the number of UK-based physics graduates increased by 7.6 per cent between the summers of 1991 and 1992.

For physical science as a whole (which also includes fields such as

chemistry and geology), the increase in graduates was even greater, at 11.1 per cent.

In contrast — and more worrying — was the smaller increase of 3.4 per cent of graduates in engineering and technical subjects. And the employment situation paints a bleaker picture. Over the same period, the proportion of physical science graduates entering industry fell from 44 per cent to 37 per cent, a bigger decrease than that experienced by graduates in any other academic discipline, either in the arts or the sciences.

UK-based graduates from British universities 1989–92
(in thousands)



This fall was partly compensated for by the increased proportion (32 per cent compared to 28 per cent) entering careers in commerce. Nevertheless, by the end of December last year, 12.3 per cent of physical science graduates were still unemployed (compared, for example, with only 9.7 of social science graduates).

This overall trend is reflected in figures being reported by industry itself. Last week, for example, the Chemical Industries Association (CIA) produced its annual survey of graduate recruitment, which showed that the number of graduates recruited by the industry fell by 16 per cent between 1991 and 1992.

In the near term, the difficult economic climate means that the prospects for science graduates remain bleak. The CIA survey says that companies expect their recruitment to fall by a further 24 per cent this year. But, in the longer term, 90 per cent said that they believed demand for graduates would either improve or remain static, and almost half expected the demand for certain types of graduates to increase.

David Dickson

Time running out for the last US fast breeder reactor

Idaho Falls, Idaho. Managers responsible for the operation of the only fast breeder nuclear reactor left in the United States are trying to persuade the Clinton administration to shelve plans to close it down next month. They argue that the reactor should stay critical for a further 3–5 years to support an associated experiment in actinide recycling.

The survival of the Integral Fast Reactor (IFR) in Idaho would represent a setback for environmentalists, who persuaded President Bill Clinton to promise its closure last winter as part of his budget proposals for the fiscal year 1994. Clinton made the promise on the basis that the fast reactor was not close to commercial application and that the money saved by closing it could be quickly transferred into renewable energy research. The Integral Fast Reactor programme, as its name implies, seeks to integrate an existing fast reactor with a compact fuel recycling process.

The Department of Energy (DoE) is now negotiating with both the administration's Office of Management and Budget and possible Japanese backers about ways of keeping the programme alive. It is expected to file revised budget proposals to Congress for the IFR, which is located at the department's Argonne West laboratory.

Under the original budget proposal for the IFR for next year, \$60 million was to be spent on closing down the reactor, and a further \$15 million on continuing an experiment in recycling its fuel. The revised pro-

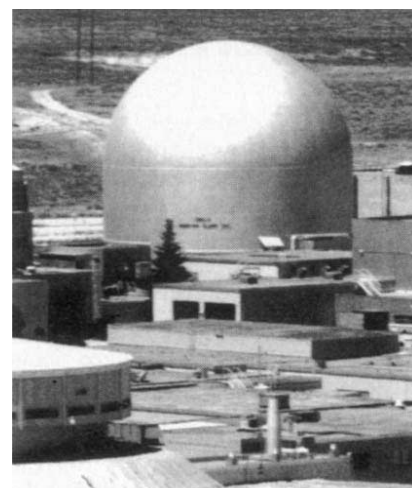
posal will acknowledge that closure would cost around \$100 million during the 1994 fiscal year, which begins on 1 October.

According to IFR managers, the DoE now believes it would be better to keep the reactor running, as that would simplify and strengthen the recycling experiment without much additional cost. John Sackett, director of IFR operations at Argonne West, says that a phased shutdown of the reactor over five years, combined with a full demonstration of the associated recycling process, would cost \$440 million.

This compares with a cost of \$400 million over the same period if the reactor was closed next month. Under the DoE's original proposal — widely criticized as impractical — irradiated fuel from foreign fast reactors would be shipped to and from Argonne West for experimental reprocessing (see *Nature* 362, 683; 1993).

It is not clear whether the administration's budget amendment will specify a closure date for the sodium-cooled, metal fuel reactor at Argonne West. A spokeswoman for the energy department said that it could not comment on budget negotiations. But the amendment will probably just raise the request to around \$100 million, and leave Congress to decide if it wants to order the reactor's shutdown.

The House of Representatives has already voted for such a shutdown, stripping out the \$15 million actinide recycling programme as well (see *Nature* 364, 6; 1993). But the Senate energy and water appropri-



IFR: another reprieve?

tions subcommittee is likely to support the continuation of the IFR programme when it considers the budget later this month, and this is likely to be approved by the full Senate.

IFR's fate will then depend on the outcome of a conference between the House and Senate. As with the Superconducting Super Collider — whose fate is likely to rest with same conference — supporters of the IFR can draw comfort from the fact that the make-up of the conference will reflect the conservative, pro-nuclear bias of the Senate.

At Argonne West, engineers are installing the purpose-built equipment which would recycle fuel from the 29-year-old experimental fast reactor (EBR 2) next door. The equipment would strip and process metal fuel rods by hydrolysis under remote control inside a chamber — built in the 1960s to accommodate a previous, long-since abandoned reprocessing scheme — surrounded by five-foot-thick concrete and glass walls.

Sackett and his team argue that most of the world's fast breeder reactor programmes took a wrong turning in the 1960s when they pursued oxide-based fuels, which do not breed well and are complicated to reprocess. The EBR 2 uses metal fuel, and the associated recycling process is far simpler than the complex chemical plant needed to reprocess oxide-based nuclear fuels.

The IFR's end-products would include plutonium mixed with other actinide elements and therefore unusable in bomb-making, they say. But the radiation inside the chamber will be so intense that electric motors used to activate equipment, for example, will last only a few months.

Japanese electricity generators have pledged \$43 million towards the Integrated Fast Reactor programme for the period 1991–96. The DoE is now seeking a further \$60 million over four years from Japan to help keep the programme going. But no agreement is expected before Congress decides on the IFR's future. **Colin Macilwain**

Reprieve for Canadian research

Ottawa. Mrs. Kim Campbell, the Canadian Prime Minister, has reversed a decision announced by the federal government last month that would have virtually halved the funding of a research programme launched in 1989 to increase the country's international competitiveness.

Campbell, whose conservative government is about to face a federal election, was reacting to a wave of protest from both the research and industrial community that had greeted a decision to cut funds for its Networks of Centres of Excellence (NCE) programme from C\$60 million to C\$30 million a year (see *Nature* 364, 566; 1993).

Addressing a meeting of the Rotary Club of Toronto, Campbell said that both she and the Minister for Science and Small Business had listened closely to criticism of the decision. As a result, she said, funding for the programme will be maintained at C\$48 mil-

lion a year for each of the next four years.

The NCE programme currently supports about 800 research workers, as well as almost 2,000 graduate students and postdoctoral fellows, and covers fields ranging from biotechnology to robotics. Campbell said that restoring the funds that were to have been cut "will be done within existing resource levels and without affecting the funding of other science and technology activities of the government".

But she also criticized that low level of industrial contribution to the programme, describing the C\$4 million that the private sector has provided so far as "paltry" and unacceptable. Campbell repeated previous calls that business and industry should join the government in both in becoming partners in funding research and development and in reducing the national deficit.

David Spurgeon

Museum cuts spark protest

Ottawa. A major restructuring of the Canadian Museum of Nature and the decision to lay off about 20 per cent of its staff are causing concern among environmental organizations, museum professionals and individuals.

Staff cuts announced this summer include nine researchers (seven scientists and two assistants). Others who will lose their jobs are six collection experts, three library staff, and thirteen members of the education and didactic division, as well as six individuals in business operations and publishing.

The changes at the museum are taking place following revisions to its mandate that were introduced when the museum — formerly part of the National Museums of Canada Corporation — became a separate Crown corporation in 1990.

The museum's director, Alan R. Emery, says that its new mandate, which is similar to that now being followed by Britain's Natural History Museum and similar institutions in other countries, means that all research can no longer be strictly curiosity driven, and that as a result it is being reorientated away from primarily disciplinary interests.

Emery has therefore created a series of multidisciplinary 'programmes' based on broad issues considered to be of interest to both science and society, rather than what he describes as "the simple survey type of work that was traditionally done in this institution".

Another revision to the museum's mandate has been the requirement that it should become more commercially minded. In particular, says Emery, the museum is now

required to seek funding through admission charges to the public, and through private sources. In order to encourage what he describes as a "business thrust", it was given the legal right to earn money and to keep it to support its programme, rather than returning it to the Crown as previously.

Yet although the mandate of the museum has been enlarged, its public funding has been reduced. The current budget of C\$18 million is about C\$3 million less than it was four years ago; in the meantime, the museum's mandate has been enlarged. And Emery says the staff cuts are a direct result of the fall in federal funding.

Following the announcement of the redundancies, the museum staff has requested the minister of the new Heritage Department, formed recently by Mrs Kim Campbell, the Prime Minister, to order a stay on restructuring and an investigation of the museum's management.

Their demands are being supported by an environmental network, called "True Friends of Nature", which is being coordinated by the Sierra Club. The network has appealed to the public to "save" the museum, which, it says, "is experiencing a serious and profound crisis".

The network also claims that "fundamental flaws inherent in the governance of the museum ... are in large part responsible" for the changes. It says too much power is concentrated in the hands of the director and chairman of the board of trustees, and there is no participation in museum policy by interested groups such as itself and by the museum's staff.

David Spurgeon

Biotech finds new voice

Washington. Health-care companies in the state of California, home to more nearly 30 per cent of the US biotechnology industry, hope to be able to speak with one voice on key issues of public policy such as President Bill Clinton's proposed reforms with the recent formation of a new California Healthcare Institute.

Located temporarily at the offices of Genentech Inc. in South San Francisco, the new institute plans to set up a permanent office in San Diego. It currently has 59 members drawn primarily from the biotechnology, medical device and pharmaceutical industries, although academic institutions are also eligible to join.

The president's proposals for health-care reform, which are expected to be announced in outline form later this month, are at the top of the institute's agenda. But its officials also plan to consider problems ranging from the disposal of low-level radioactive waste

to future funding levels for the state's universities and other educational establishments.

Charles C. Edwards, former president and chief executive officer of Scripps Institutions of Medicine and Science, has been chosen to head the institute. Edwards is no stranger to the workings of Washington, having served on numerous government advisory panels, and as commissioner of the US Food and Drug Administration between 1969 and 1973.

One of the first tasks of the institute has been to conduct a survey of 138 of the state's largest health-care companies, in an attempt to quantify the economic contribution and importance of the industry to California. Full details of the survey are expected later this month. Preliminary findings indicate that the biomedical companies founded since 1988 account for 10 per cent of the state's new jobs during this period.

Diane Gershon

US wetlands plan meets with a mixed reaction

Washington. The Clinton administration has announced a new policy that attempts to end the debate over the "wetlands" of the United States — areas that include swamps, ponds, estuaries and other watery habitats that support many endangered species and naturally control pollution and floods. In doing so, however, it has still failed to clarify precisely what the term means.

Government estimates show that the United States is losing about 300,000 of its total 270 million acres of wetland habitat each year. In a series of attempts to halt this process, both the Reagan and Bush administrations have continually revised their wetlands policies over the past decade.

The result, however, was to leave scientists, conservationists, developers and land-use officials squabbling over which lands should be protected. In the process, definitions have become so unclear that many of the nine federal agencies that oversee wetlands now use vastly different standards in qualifying them for protection.

The Clinton administration has now announced what it called a "balanced, common-sense, workable set of improvements" for wetlands. One aim is to eliminate loopholes that have allowed developers to build shopping malls, office buildings and housing tracts in certain wetlands areas. Also, Clinton's plans would reverse a Bush administration proposal that would have allowed development on 1.7 million acres (or one per cent) of Alaska's wetlands, including 345,000 acres of fragile coastal areas.

Nonetheless, the NWF and other conservationist groups have still attacked most of Clinton's other wetlands directives, claiming that they make too many concessions to developers and farmers. For example, they criticize the plan's call for "mitigation banking" by which a wetland may be developed if another wetland preserve is created elsewhere.

The new plan also fails to bridge what many see as a gap between science and politics, by leaving the task of crafting a precise and lasting definition of the concept of wetlands to a scientific committee formed by the National Academy of Science's National Research Council.

The 18-member committee will hold its first meeting in Washington later this month. Its task is to study regional differences among wetlands, and discuss what levels of water cover, soil moisture and vegetation should be used in delineating the habitats.

The committee is due to complete its recommendations in September 1994. Until then, the various federal agencies overseeing wetlands will continue to follow patchworked standards in carrying out the administration's new policies.

Susan Greene

MIT's Amazon outpost

São Paulo. The US Massachusetts Institute of Technology (MIT) has agreed to provide technical backing for a US\$5-million project designed to stimulate the growth of a biotechnology industry based on the biological and genetics resources of Brazil's Amazon region.

An agreement to collaborate was signed last month between the institute, the governor of the state of Amazonas and the director of a newly created biotechnology laboratory, the Centro de Biotecnologia da Amazônia (CBA), in the state capital Manaus.

Under the terms of the agreement, whose initial phase will last for three and a half years, MIT researchers from disciplines such as microbiology, enzymology and biochemical engineering will help to develop new techniques appropriate to the resources and economic interests of the region.

Initial activities will concentrate on areas such as agriculture, fishing and cattle breeding. At a later stage, however, attention will also be given to the more controversial area of screening the Amazon region's flora and fauna for pharmaceuticals.

MIT officials compare the potential significance of the new agreement to that provided several decades ago by the institute to the state of São Paulo, which helped to create Brazil's now flourishing aircraft construction industry.

Brazilian scientists say that one important characteristic of the new initiative is that, unlike deals struck by other countries with foreign companies, it will include agreement that any commercially important genetic resources discovered in the region will be developed locally, rather than outside Brazil.

"The idea is not to do what Merck did to Costa Rica", says Isaias Raw, head of the Butantan Institute of São Paulo. Referring to a deal with the pharmaceutical company

Inbio, he compares Costa Rica to the woman played by Demi Moore in the film *Indecent Proposal*, who agrees to sleep with actor Robert Redford for \$1 million.

"Just cataloguing plants will not lead you anywhere", says Raw. "Scientists in Amazonia should start using biotechnology to exploit the region's biodiversity."

The creation of the laboratory has been one of the first tasks of a new foundation, the Foundation for Conservation of the Biodiversity of Amazonia (FCBA), which has been set up at the initiative of the state governor, Gilberto Mestrinho.

In the past, Mestrinho has been strongly criticized both within and outside Brazil because of his staunch defence of the economic development of Amazonia, and for paying insufficient attention to the need to conserve the environment.

The new foundation, set up at a time when the idea of "sustainable development" popularized at last year's Earth Summit in Rio de Janeiro is on everyone's lips, is intended to counter this image. "We hope to carry on scientific and technological research within a conservationist philosophy", says Raimar da Silva Aguiar, the state's secretary for planning, who played an important part in setting up the foundation.

The stated objective of the foundation is to "keep centres of excellence directed towards seeking development alternatives for the state of Amazonas and the region". Seed money of \$500,000 has already been raised from private enterprises, including mining, construction and biomedical companies, as well as the Brazilian branches of Sharp and Semp-Toshiba.

The foundation has promised to make as much use as possible of resources already existing in the region, providing support not merely to the biotechnology centre but also local institutions such as the National Institute of Amazon Research (INPA), universities and the agricultural research units of the Brazilian Ministry of Agriculture.

The new laboratory being funded by the foundation has already been given a building in Manaus by the state of Amazonas. It will draw on the experience of researchers from the south of Brazil who have organized a similar centre at Porto Alegre, in the state of Rio Grande do Sul. Much of the impetus for its research programmes, however, will come from the visiting MIT researchers, who will assist with both teaching personnel and technology transfer.

Anthony Sinskey, associate director of the institute's Biotechnology Process Engineering Centre and one of the leaders of the project, says he hopes MIT will profit from the opportunities for research in what is relatively virgin territory, as little modern biotechnology research has been done in the region. "It should open new intellectual

questions and frontiers in science and engineering", says Sinskey.

According to Brazilian scientists, the foundation is expected to take the largest share of any royalties and patents derived from research in the laboratory. "But obviously MIT will have a share in things developed jointly", says Diogenes Santos, the founder of the biotechnology centre in Porto Alegre.

FCBA officials say they are open to cooperation agreements with other foreign institutes besides MIT. But they insist that the research has to be carried out in Brazil. Many remember the country's experience with rubber, which is native to Amazonia, but which was exploited in other parts of the world. "If you do not do research in the country, nothing stays here," says Raw.

Ricardo Bonalume Neto

Money-saving space station plan

Washington. US and Russian space agencies have agreed to work together on developing a joint international space station which that incorporate the main elements of current designs for both the US Freedom station and the proposed Russian Mir 2.

Officials at the US National Aeronautics and Space Administration (NASA) say that the joint station, plans for which are to be produced by 1 November, could use Mir as an initial building block. Such a move would speed up the overall programme by two years, and as a result could save the United States a substantial amount of money.

But the agreement, signed in Washington last week by US vice-president Albert Gore and Prime Minister Victor Chernomyrdin, also means that recently completed plans for a redesigned US space station — due to be presented by NASA to President Bill Clinton this week — will be subject to still further extensive revision in November.

Fortunately for NASA, details of the US-Russian space station will not be ready until after Congress has approved funding for the US space station for the coming fiscal year. It is the details that will determine which NASA contractors are likely to lose work to Russia under the deal, and such knowledge could result in lost votes for the programme.

The existing partners on the US space station — Europe, Canada and Japan — are being kept informed of the progress of discussions. But they are not being directly involved in negotiating the deal, and will effectively have to accept it as a *fait accompli*.

The United States has also promised to pay Russia \$400 million over the next four years for using existing Russian facilities in cooperative space programmes. Programmes in which the two countries plan to collaborate include sending US astronauts to the existing Mir 1 station, and developing a joint space suit.

Colin Macilwain

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Genetic resources for local industry?

Molecular constructivity

SIR — Dan Graur accused us in your pages of “molecular deconstructivism”. But only his letter¹ is destructive, because it failed to address the conceptual issues of our paper². Instead, Graur limits himself to criticizing our terminology. Moreover, in an apparent attempt to ridicule us, he then “invents”, but imputes to us, additional terms and applications that we clearly excluded from consideration, thus grossly distorting our effort. Most scholars will agree that our nomenclature was not intended either to amuse ourselves or to vex our colleagues, but rather to identify and explain concepts that now have no names but are important for understanding the evolution of genomic sequences and structures.

First, we addressed the need for a more general term for any definable stretch of polynucleic acid (nuon). As recently brought to our attention, Nei and Tajima responded to the need for a similar definition more than a dozen years ago when they proposed the term nucleon (borrowed from nuclear physics) to describe definable nucleic acid sequence in a more general way³. Unfortunately, the term did not take hold, perhaps because another field already used the same name in such a different sense.

Second, the concepts of potaptation, exaptation, adaptation and nonaptation, and in particular the cross-convertibility

between these different stages, with resulting increase of the genomic flux, increasingly prove to illustrate vital evolutionary principles. It is therefore logical (and helpful) to integrate these concepts into aspects of the genomic nomenclature (see figure).

This allows us, for example, to give non-genic DNA elements (formerly considered junk DNA as in the case for middle repetitive elements) the attention that they deserve, given their role in shaping future genes or parts thereof including their regulation⁴.

Perhaps we should envy physics, where it appears that novel concepts and their accompanying nomenclature receive serious attention rather than anti-intellectual ridicule.

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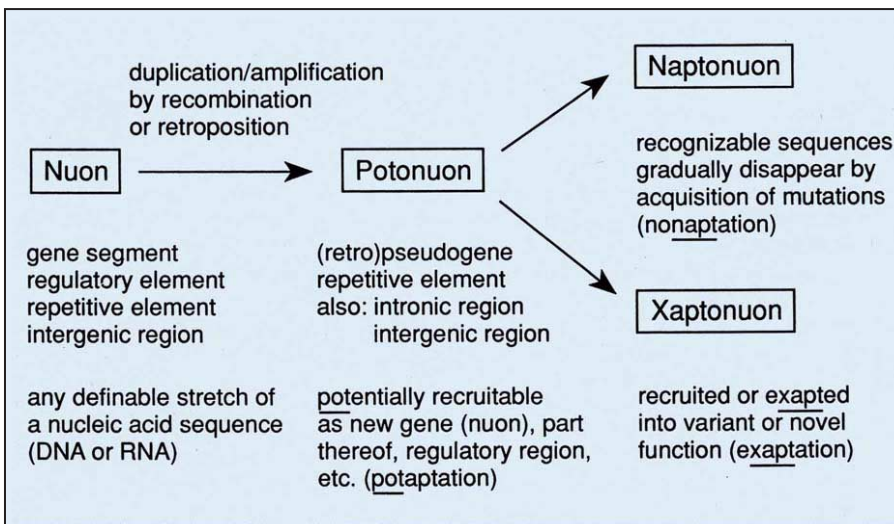
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Chromosome segments, including entire genes, are not the only elements that can be amplified within a genome. Repetitive elements, for example, that are not considered genes but can be defined as nuons, are generated by efficient amplification, for example, through retroposition. The new nuon may have a variety of impacts on a possible neighbouring gene including its expression. Each nuon, therefore, has the potential to become part of an adjacent gene, including, but not limited to a new acceptor or donor site for splicing, part of a new exon, transcriptional enhancer or polyadenylation signal. As a result of this potential, a new nuon is always a potonuo. If indeed exapted into a new role, it may be termed a xaptonuo. If the recognizable sequence, as probably in most cases, disappears after a long enough evolutionary time span due to mutational attrition, it is considered a nonaptation and thus a naptonuo. The latter scenario, however, does not preclude the later exaptation of such a neutral drifting sequence into a new role: An intronic sequence, for example, may be recruited as a novel exon in an existing gene⁵.

No to “genethics”

SIR — The provocatively titled “New genetics means no new ethics”¹ makes the important point that society does not need new ethics to cope with the impact of genetic technology. There is no inherent clash between genetics and human values as some books, including one under the title *Genethics*², would like to have us believe. This concept should be stopped because almost all the issues raised by application of genetics are not novel.

What is needed is a revival and renewed discussion of ethical values as society interacts with technology, and reassurance that scientists are responsible, as we have seen in recent issues of *Nature*^{3–5}. Applications of genetics have been a useful catalyst for this process⁶. Sequencing of the human genome may not create new ethical dilemmas, or necessarily make them worse, as your leading article points out, but the sheer number of disease-related genes identified will make the number of such dilemmas greater. The number of known “early-death” related genes will increase so that it may even be impractical for insurance companies to identify individual risk, let alone the ethical arguments⁷. Life will be more complex, even the supposedly simple case of imposing higher insurance fees on smokers will become more cloudy should we find strong genetic determinants for drug addiction, to add to the environmental determinants we already know.

In the final paragraph of the article, Maddox concludes that it is unwise for geneticists to say “never touch the germline”. Although geneticists may like to say this, perhaps naively to reassure the public that they have nothing to worry about, over the past two or three years “ethicists” have been seriously revisiting the issue of human germline genetic manipulation⁸. Some want a ban on germline manipulation, but the somatic cell/germline division is less important than the therapy/cosmetic border. In the easy cases of severe disease, safe and nonexpensive germline gene therapy can make sense. We should encourage discussion of these complex issues and, in the real world, never say never.

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When entropy does not seem extensive

Earlier speculations about the entropy of black holes has prompted an ingenious calculation suggesting that entropy may (in special circumstances) be the same inside and outside an arbitrary boundary.

EVERYBODY who knows about entropy knows that it is an extensive property, like mass or enthalpy. That, of course, is why the entropy of some substance will be quoted as so much per gram, or mole. If you then take two grams, or two moles, of the same material under the same conditions, the entropy will be twice as much. And there should be no confusion about the units; the simple Carnot definition of a change of entropy in a reversible process is the heat transfer divided by the absolute temperature, so that the units of entropy are simply those of energy divided by temperature, joules per degree (kelvin) in the SI system. The definitions of the Gibbs and Helmholtz free energies would be dimensionally discordant for that reason were it not that entropy (S) always turns up multiplied by temperature T . So much will readily be agreed.

Of course, there is more than that to entropy, which is also a measure of disorder. Everybody also agrees on that. But how is disorder measured? By the number of ways in which the constituents of some material (the atoms and molecules) can be rearranged without changing its properties and without energetic consequences. But now there comes a snag.

Like any extensive property, the combined entropy of two separate chunks of material should be the *sum* of the two entropies, but the number of rearrangements of the combined system must be the *product* of the numbers of ways in which the two parts separately can be rearranged. How to reconcile that with extensivity? By supposing entropy is proportional not to the number of rearrangements (technically called 'complexions'), but with the logarithm thereof. And because entropy decreases as disorder increases, the constant of proportionality must be a negative (real) number.

From that it follows that $S = S_0 - K \log N$, where K is a positive constant with the dimensions of entropy, N is a number (without dimensions) measuring disorder and S_0 is an arbitrary constant entropy. All that is simply a precis of the standard introductory chapter in statistical mechanics textbooks, most of which go on to show how to calculate the properties of assemblages of, say, diatomic molecules from a knowledge of their individual behaviour. Because the number of complexions of a particular state of an assemblage is invariably a function of the number (n) of molecules it contains, usually in the form of $n!$, because n is usually large and because $\log(n!)$ can then be approximated by $n \log n$, the extensive

property of entropy then follows simply from the appearance of the leading factor n : entropy is proportional to the number of molecules.

That is what the textbooks say. It also makes sense of what is known of the thermodynamics of the real world. In a sample of a diatomic gas, for example, there are vibrations (one) and rotations (two) as well as three rectilinear degrees of freedom. But the problem is to tell how the energy available is distributed among the different degrees of freedom. The arithmetic simplifies marvelously because (in this case) each molecule and each of its degrees of freedom is independent. The best measure of disorder works out at $N = Z^n$, where n is the number of molecules, and where Z , which must be a function of the temperature, is the single-molecule partition function. (The arithmetic, in this case, is further simplified because Z itself factorizes into separate components for each degree of freedom, which then appear additively in the final entropy.) Unsurprisingly, the constant K is usually identical with nk , where k is Boltzmann's constant. So the extensivity of entropy seems inescapable.

So why is the entropy of a black hole proportional to the square of its radius, and not to the cube of it? To its surface area rather than to its volume? That question is not, of course, the one most likely to pop into the mind at this point in this recitation. The idea that black holes should have an entropy at all (due to Stephen Hawking) may seem bizarre until one reflects that they exchange energy and mass with the world outside them, so that the Carnot rule applies. Because black holes also evaporate, generating particulate radiation in the surrounding space, their entropy can be equated with the calculable entropy of that radiation, whence the result that the entropy is proportional to the surface area.

This is precisely the question that seems to have provoked Mark Srednicki, now at the University of California at Berkeley, into a formal consideration of why that may be (*Phys. Rev. Lett.* **71**, 666; 2 August 1993). The outcome may be inconclusive, but the argument has all the attractions of being the nearest thing to a dimensional argument that a respectable physicist would wish to publish, and of being suggestive and stimulating as well.

A bit of quantum mechanics goes into the argument as well, notably the notion of the density matrix — an artificially constructed operator (on quantum states) that is

well suited to the discussion of systems in which one part (say the black hole) is singled out for attention while the remainder (the Universe outside it) is dealt with in less detail, perhaps because some averaging process is appropriate, or because the whole problem may not be calculable at all. (In Dirac's notation, the density matrix corresponding to some state of the whole Universe would be represented as $|1\rangle\langle 1|$, where "1" is simply the name for a particular state of the Universe.) What matters, where entropy is concerned, is that the density matrix, like all matrices, has eigenvalues from which the entropy can be calculated.

So imagine that the Universe is partitioned into two parts by means of a closed boundary of some kind and filled with a scalar field such as the sound field within a solid. It is then possible to deal with the density matrix in two distinct ways — by concentrating on what is inside the boundary and averaging over all the degrees of freedom that lie outside and, conversely, by concentrating on the degrees of freedom that lie outside and averaging over the others. But then, the argument goes, the two matrices must have the same eigenvalues except that the one with the larger number of dimensions will have extra zeros (which do not contribute to the entropy). So the inside and the outside must have the same entropy, which can then only depend on their sole shared property, the boundary between them.

Srednicki also deals with a somewhat special case (which is why he does not solve the black-hole problem), that of the ground state of a system of harmonic oscillators whose vibrations are coupled energetically to each other. He arbitrarily separates them into two and confirms the qualitative dimensional arguments he has already advanced. It is necessary, along the way, to get rid of the infinities arising from the tendency of the results to diverge to infinity at the low- and high-frequency ends of the spectrum, but, for good measure, Srednicki set about confirming the results by computation. Entropy does vary with the square and not the cube of the linear dimensions of lattice carrying oscillators.

For the time being, none of this argues for the abandonment of the doctrine of extensivity. It is more important that this exercise seems to show that the thermodynamics of black holes may yet be dealt with explicitly, as other entropy calculations are made. And that could be exceedingly important.

John Maddox

Snappy exocytotoxins

Wieland B. Huttner

THE viciousness of neurotoxins has its bright side, for they have been powerful tools in the molecular dissection of the machinery mediating synaptic transmission. In many classical studies, they were the essential probes that led to the identification of key molecules, for example certain neurotransmitter receptors and ion channels. More recently, with the increasing number of cloned proteins that are often first characterized in molecular but not functional terms, neurotoxins have been used to demonstrate that an already identified protein indeed functions as suspected. One such case is reported by Blasi *et al.* on page 160 of this issue¹, and concerns a protein involved in exocytosis.

The clostridial neurotoxins, tetanus toxin and botulinum toxin, are responsible for tetanus and botulism respectively. They were known to be potent inhibitors of neurotransmitter release, but until about a year ago it was not clear exactly how this inhibition is caused. A breakthrough occurred when Montecucco and co-workers^{2,3} showed that tetanus toxin is a zinc-dependent endopeptidase that selectively cleaves the synaptic vesicle membrane protein VAMP/synaptobrevin, a finding that was soon afterwards also reported by Jahn, Niemann and collaborators⁴.

This observation indicated that VAMP/synaptobrevin, as previously suspected,

indeed has a crucial role in neurotransmitter release, presumably through involvement in the exocytosis of the synaptic vesicle. Moreover, Schiavo *et al.*³ not only studied tetanus toxin but also three of the seven serotypes of botulinum toxin, botulinum neurotoxins A, B and E. Whereas botulinum neurotoxin B cleaved VAMP/synaptobrevin at the same single site as tetanus toxin, botulinum neurotoxins A and E had no effect on this protein. But because the A and E neurotoxins are nonetheless zinc endopeptidases, this raised the possibility that they might cause inhibition of neurotransmitter release by proteolysis of proteins other than VAMP/synaptobrevin.

Blasi *et al.*¹, and independently Montecucco and co-workers (G. Schiavo *et al.*, manuscript submitted), now show that this is indeed the case. They report that inhibition of neurotransmitter release by botulinum neurotoxin A is associated with the selective cleavage of SNAP-25 (synaptosomal-associated protein of M_r 25K); a large part of SNAP-25 seems to be localized to the plasma membrane, and the protein is not only implicated in synaptic vesicle exocytosis but is also a key player in constitutive vesicle exocytosis in neurons (see ref. 5 for review).

As was the case for cleavage of VAMP/synaptobrevin by tetanus toxin and botulinum neurotoxin B, the selective cleavage of SNAP-25 by botulinum neurotoxin A

provides direct evidence that SNAP-25 participates in neurotransmitter release, and supports the concept that the same protein family (SNAP-25 has two isoforms) operates in both constitutive and regulated exocytotic processes. Yet what is even more gratifying is that with SNAP-25 a second protein component of the docking/fusion complex described by Rothman and colleagues⁶ has turned out to be a target for a clostridial neurotoxin that inhibits neurotransmitter release.

But there is more to come. Besides SNAP-25 and the soluble proteins NSF (*N*-ethylmaleimide-sensitive factor) and α - and γ -SNAPs (soluble NSF attachment proteins), the protein complex discovered by Söhlner *et al.*⁶ contains two membrane proteins — VAMP/synaptobrevin (as v-SNARE of the vesicle to be docked and fused) and syntaxin (as t-SNARE of the specific target membrane); syntaxin has been implicated in the docking of synaptic vesicles at presynaptic active zones (see ref. 7 for review). In a separate study Jahn, Niemann and co-workers⁸ will show that another serotype of botulinum toxin, botulinum neurotoxin C, selectively cleaves syntaxin, and thus a third component of the putative docking/fusion complex, and that cleavage correlates with the inhibition of neurotransmitter release caused by the toxin.

What about the other serotypes of botulinum toxin? As it turns out, except for botulinum neurotoxin G, specific target proteins for each of the other botulinum neurotoxins recently have been, or soon will be, reported by Montecucco and co-workers (ref. 9; G. Schiavo *et al.*,

Target proteins of clostridial neurotoxins

Neurotoxin	Target	Cleavage site	Cell type	Intracellular localization	Reference
Tetanus toxin	VAMP/synaptobrevin*	Gln76–Phe77	Neuron	Synaptic vesicle	2–4
	Cellubrevin	Not identified	All cells	Vesicles of the endocytosing/recycling system	14
Botulinum neurotoxin A	SNAP-25	Near C terminus	Neuron	Presynaptic plasma membrane, elsewhere?	1, G. Schiavo <i>et al.</i> [‡]
BoNt/B	VAMP/synaptobrevin*	Gln76–Phe77	Neuron	Synaptic vesicle	3
BoNt/C	Syntaxin	Near C-terminal membrane anchor, cleavage generates soluble fragment	Neuron	Presynaptic plasma membrane, elsewhere?	8
BoNt/D	VAMP/synaptobrevin*	Lys59–Leu60 Ala67–Asp68	Neuron	Synaptic vesicle	G. Schiavo <i>et al.</i> [‡] S. Yamasaki <i>et al.</i> [‡]
	Cellubrevin	Not identified	All cells	Vesicles of the endocytosing/recycling system	S. Yamasaki <i>et al.</i> [‡]
BoNt/E	SNAP-25	Not identified	Neuron	Presynaptic plasma membrane, elsewhere?	G. Schiavo <i>et al.</i> [‡]
BoNt/F	VAMP/synaptobrevin*	Gln58–Lys59	Neuron	Synaptic vesicle	9, S. Yamasaki <i>et al.</i> [‡]
	Cellubrevin	Not identified	All cells	Vesicles of the endocytosing/recycling system	S. Yamasaki <i>et al.</i> [‡]
BoNt/G	?				

*Cleavage sites refer to isoform 2. [‡]Work submitted for publication.

manuscript submitted) and Jahn, Niemann and collaborators (S. Yamasaki *et al.*, manuscript submitted). Interestingly, these are the same proteins that have already been identified as the targets of the other clostridial neurotoxins. Thus, botulinum neurotoxin D cleaves VAMP/synaptobrevin (though at different sites to those attacked by the type B neurotoxin); E cleaves SNAP-25; and F again cleaves VAMP/synaptobrevin (again at a different site) (see table).

There are several intriguing points about this story as it approaches completion. First, the fact that the sum of the identified clostridial neurotoxin targets covers all of the membrane-associated/transmembrane proteins shown to interact with NSF/ α -SNAP/ γ -SNAP is an extraordinary congruency of experimental evidence. It suggests that the affinity purification approach⁶ has revealed the complete set of the proteins that make up the 'constitutive' core of the synaptic vesicle docking and fusion machinery. ('Constitutive', because proteins with significant homologies to VAMP/synaptobrevin, SNAP-25 and syntaxin of neurons exist in yeast, where they are involved in docking/fusion events at various sites in the secretory pathway; for review see refs 7, 10).

Second, and implicit in the first point, it is remarkable that none of the proteins implicated in the 'regulated' aspect of neurotransmitter release and synaptic-vesicle mobilization, docking and fusion, because of their selective association with synaptic vesicles (synapsins, rab3A, synaptophysin) and their calcium-binding properties (synaptotagmin) (see refs 7 and 11 for reviews), has been found to be a target for the clostridial neurotoxins. That is perhaps not so surprising as far as synaptotagmin is concerned, as several lines of evidence suggest that this protein probably operates as an inhibitory modulator of regulated (calcium-dependent) exocytosis rather than as an essential component of the docking and fusion machine (see ref. 12 for review). Thus clostridial neurotoxins have chosen their targets with insight, attacking the constitutive core rather than the regulated accessories of the docking and fusion

machine.

For all the progress that has been made, major questions are yet to be resolved. For example, synaptic vesicles exist in at least two functional populations which can be separated by subcellular fractionation¹³ — those that are docked at the active zones and those that are not. Do the clostridial neurotoxins attack their target proteins in a differential manner depending on whether or not vesicles are docked? In their work on SNAP-25, Blasi *et al.*¹ observe cleavage by botulinum neurotoxin A in a synaptosome-derived 12,000g membrane fraction, which largely contains presynaptic plasma membranes and docked synaptic vesicles. On the other hand, in their work on syntaxin⁸, they show cleavage of this protein in a fraction of light membranes which are not sedimented at 12,000g and which consist primarily of free synaptic vesicles. This is remarkable, because syntaxin is thought to reside in the presynaptic plasma membrane at the active zones. These findings

call for a thorough analysis both of the precise localization of syntaxin within the nerve terminal and of its susceptibility to toxin-catalysed cleavage in relation to its intracellular localization.

Above all, however, it should be realized that whereas the key components of the synaptic vesicle docking and fusion machine have been identified, their exact roles in the cascade of events leading to exocytosis have yet to be delineated. Do VAMP/synaptobrevin, SNAP-25 and syntaxin mediate docking, or fusion, or both? To obtain answers to these questions, clever biochemical assays will be required to dissect the overall process of synaptic vesicle docking and fusion into its individual steps. The clostridial neurotoxins will no doubt be tremendously helpful in this task. □

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CHEMISTRY

Reactions à la mode

Richard N. Zare

MANY chemical reactions can yield more than one product, some useful, others a nuisance. Can chemists control the branching ratio of the product yield by the way in which the reactants are excited? Another important step in answering this long-standing practical question has just been reported by Metz and colleagues¹. In the bimolecular reaction of partly deuterated water (HOD) with hydrogen atoms, they were able to break either the O–H or the O–D bond by selectively exciting each of them into higher vibrational states.

The gas-phase reaction is the elementary one shown below: here thermal hydrogen atoms from a microwave discharge through a mixture of H₂ and He react with



labelled water molecules, HOD, having two different isotopes of hydrogen. The products are distinguished only by the replacement of normal hydrogen (H) by heavy hydrogen (D). These isotope markers serve to indicate which side of the water molecule reacts. If the water molecule is unexcited, both sides react to roughly the same extent². But the O–H and O–D bonds have, of course, different vibrational frequencies and by laser excitation of the gas-phase mixture tuned to either an O–H or O–D vibrational over-

tone, Metz *et al.* prepared HOD in one or the other of two nearly isoenergetic, highly vibrationally excited states. One state had four quanta of excitation in the O–H stretch ($4\nu_{\text{OH}}$ with $13,800\text{ cm}^{-1}$ of vibrational energy), the other had five quanta in the O–D stretch ($5\nu_{\text{OD}}$ with $12,800\text{ cm}^{-1}$ of vibrational energy).

In both cases, the bond that was initially excited reacted preferentially. In particular, Metz *et al.* found that exciting $\text{H} + \text{HOD}$ with $4\nu_{\text{OH}}$ favours breaking the H–O bond by a factor of approximately 200 over the O–D bond, and that exciting the mixture with $5\nu_{\text{OD}}$ favours the reverse by about a factor of 220. Vibrational excitation of the HOD reactant can be used to control the $\text{H} + \text{HOD}$ reaction to produce almost exclusively either the $\text{OD} + \text{H}_2$ or the $\text{OH} + \text{HD}$ products.

This work extends earlier studies³ that first demonstrated site-specific chemistry in the $\text{H} + \text{HOD}(4\nu_{\text{OH}})$ reaction in which just the O–H bond was broken selectively. By preparing $\text{HOD}(5\nu_{\text{OD}})$, Metz *et al.* have now shown that this control can be used to select either product channel. Such behaviour was predicted almost ten years earlier⁴, but the experimental realization of this effect has been seen only in the past year. In the same reaction carried out under different conditions of reagent energization, hypothermal H atoms reacted with HOD having one quantum of stretch excitation in either the H–O or O–D bond, which was prepared

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by infrared laser excitation, and the product branching ratio was found to be controlled almost quantitatively by the choice of bond excited². This work set limits of greater than 25:1 and less than 1:6 for the selectivity of the two product channels. The results of Metz *et al.* for more highly vibrationally excited HOD give a still more striking degree of control in product selection.

To appreciate the significance of mode-selective chemistry, it is useful to recall that most chemical reactions are accelerated by heating, but in general internal energy is equilibrated within the molecule, so the energy deposited in a specific mode is rapidly redistributed into other isoenergetic modes — it is hard to persuade it to stay put. The H+HOD system clearly demonstrates that counterexamples exist, but what determines whether these effects can be observed?

It seems that three conditions are necessary: first, it must be possible to excite a state of the reactant (an eigenstate or a superposition of eigenstates) that localizes energy in some part of the molecule; second, this energy must remain localized during the reaction; and third, this prepared state must enhance reactivity of that portion of the molecule.

How far can theory help? Theoretical groups are active in calculating the H+HOD reaction using both semi-classical⁵ and quantum mechanical⁶⁻⁹ methods that include different internal motions of the H-HOD complex to various extents. For this simple system, they can predict qualitatively the observed mode-specific effects, which encourages us to believe that an understanding of the selectivity is in hand. Theory teaches us that in general those vibrational modes that are most effective in promoting a reaction are those that involve motions along the 'reaction coordinate', that is, motions in the transition state that cause the rearrangement of the nuclei from reactants to products. In the H + HOD reaction, the transition state is calculated to have a structure in which the leaving hydrogen atom (H or D) is elongated compared to the equilibrium bond length in HOD. It is not surprising then that stretch excitation promotes reaction with

the stretched hydrogen atom. Earlier experiments have in fact shown that excitation of the bending mode in water does not appear to promote reaction whereas excitation of the stretching mode can increase the reaction rate by orders of magnitude^{2,3}.

Metz and colleagues' fascinating demonstration of mode-specific chemistry begs the question of how large a system can still show mode selectivity. It isn't clear whether the effects can be significant in condensed media, where most prepara-

tive chemistry is carried out, and where energy localization during the timescale of a reaction is much more problematic. Mode-specific chemistry is certainly of intense interest to those who wish to understand how individual chemical reactions occur, but it remains to be seen whether it can change the practice of chemical transformations. □

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NONLINEAR DYNAMICS

Uncertain determinism

Eric J. Kostelich

THE ability to perform reproducible experiments lies at the heart of the scientific method. Yet on page 138 of this issue, J. C. Sommerer and E. Ott¹ discuss an example of a deterministic physical system for which reproducible laboratory experiments are intrinsically impossible. No randomness is built into the model, yet the final state of the system cannot be predicted with certainty if there is any error (no matter how small) in the measurement of the initial condition. This unusual situation arises from the existence of 'riddled' sets of initial conditions that correspond to different outcomes.

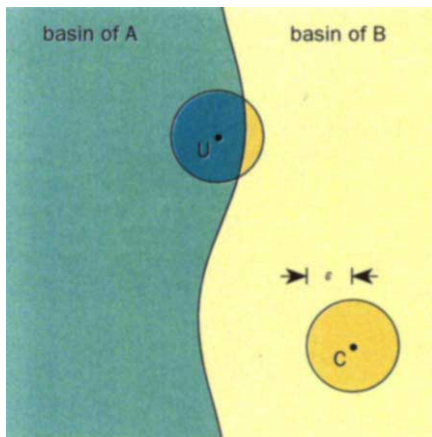
Typical nonlinear dynamical systems have several possible time-asymptotic final states, called attractors. In the case of a periodically forced pendulum that is allowed to rotate through 360 degrees, the

final states might be periodic clockwise and periodic counter-clockwise rotation; call them A and B. The initial position and velocity of the pendulum completely determine the attractor. The set of all initial conditions with the same attractor is called the basin of attraction. The figure shows a simple case where all the initial conditions on the left go to A and those on the right go to B.

Consider a laboratory experiment with such a pendulum. Even if the apparatus is a perfect realization of the mathematical model, there is some error of size ϵ in the measurement of the initial condition. If the measured point is the one labelled C, which is well away from the basin boundary, then the measurement error still allows the outcome of the experiment to be predicted with certainty. If the measured point is U, then the outcome is uncertain, because the error means there is a positive probability that the true point is in the other basin.

This simple case has two nice properties. First, although the outcome starting at a point like U is uncertain when the measurement error is ϵ , there is a smaller tolerance τ that makes the outcome certain. (Of course, τ depends on the initial condition — the closer it is to the basin boundary, the smaller τ must be to make the outcome certain. As the boundary contains no volume, it is extremely unlikely in practice that the initial condition would lie precisely on the boundary.) Second, the size of the set of uncertain initial conditions decreases linearly with ϵ . That is, if the measurement error is reduced by half, then so is the size of the total set of uncertain points.

The situation is more complicated if the boundary separating the two basins is fractal. The first property still holds: if the outcome from a particular point is uncertain when the error is ϵ , there is a smaller tolerance τ that makes it certain. The second property does not hold: the size of the set of uncertain initial conditions no



Demonstrating basins of attraction. A measurement error of size ϵ means that the true initial condition can be anywhere in the circle surrounding the measured point. Nevertheless, the outcome of an experiment starting from C is certain because all possible initial conditions within ϵ of C are in the same basin of attraction. The outcome of U is uncertain, because there is a positive probability that the true initial condition is in the other basin of attraction. In Sommerer and Ott's system, the outcome is always uncertain.

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longer scales linearly with ϵ , but as a power of ϵ that is smaller than 1. A big reduction in ϵ can lead to a negligible decrease in the size of the set of uncertain points². In one model of a kicked double pendulum³, a reduction of ϵ by a factor of 10^{10} cuts the fraction of uncertain points only by a factor of 10.

Recently, Alexander and co-workers⁴ found mathematical examples where the situation is even worse: the outcome from every point in each basin of attraction is uncertain for every $\epsilon > 0$. In other words, the attractor cannot be predicted if there is any measurement error at all in the initial condition. In principle, if the initial condition is known exactly, then so is the final state. Hence the process is deterministic. Yet among the points within ϵ of any initial condition, there is a positive volume of points from each basin of attraction. No smaller positive tolerance removes the uncertainty. Loosely speaking, each basin of attraction is full of holes at arbitrarily small scales — hence the term 'riddled' basins.

Sommerer and Ott have discovered a classical physical system with riddled basins of attraction. They consider a particle moving in the plane whose acceleration is governed by the gradient of a scalar potential, a periodic external force, and a linear coefficient of friction. The system can approach a chaotic attractor, or it can go to infinity; with probability one, an arbitrarily chosen initial condition is in the basin of attraction of one of them. However, because the basins are riddled, no sequence of even perfectly modelled experiments is reproducible: the inevitable measurement errors mean that there is always a positive probability that the particle will tend to infinity even though the system is started ostensibly in the basin of the bounded attractor, and conversely.

Although the scalar potential has a symmetry that is necessary for the creation of the riddled basins, there is otherwise nothing special about the choice of the equations of motion. The riddled basins persist for moderate changes in the parameters, and it is likely that potentials with other symmetries have riddled basins. So this is not an isolated pathological example. As Sommerer and Ott point out, the ability to predict even the qualitative outcome of classical physical systems cannot always be taken for granted. □

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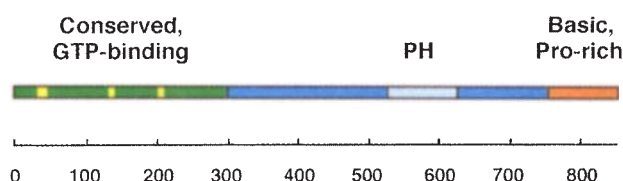
Dynamin in synaptic dynamics

Richard B. Vallee and Howard S. Shpetner

DYNAMIN received attention first as a novel type of cytoskeletal protein and subsequently as a GTPase responsible for controlling endocytosis and synaptic vesicle recycling. It has now appeared in two new guises. On page 163 of this issue¹ Robinson and colleagues identify dynamin as a synaptic phosphoprotein, providing a clue as to how the supply of synaptic vesicles in the nerve terminal may be regulated. And in developments reported elsewhere, dynamin has been found to bind to a number of Src-homology 3 (SH3)

'dephosphin'⁹). When the synaptosomes were repolarized, the dephosphin was rephosphorylated. Robinson *et al.* show that the dephosphin doublet corresponds to alternatively spliced forms of dynamin. When purified dynamin was assayed as a substrate for different kinases *in vitro*, phosphorylation by both protein kinase C and casein kinase II was observed. But phosphopeptide mapping suggests that protein kinase C is the physiologically relevant enzyme.

Of particular interest is the effect of phosphorylation on dynamin GTPase activity. Phosphorylation of the purified protein by protein kinase C (but not casein kinase II) induced a 12-fold increase in the rate of steady-state GTP hydrolysis. So depolarization of the presynaptic terminal and the concomitant dephosphorylation of dynamin would lead to a decreased rate of GTP turnover. Because the rate-limiting



Structural domains of dynamin. The amino-terminal GTP-binding domain conserved among dynamin-related proteins is shown in green, with the principal GTP-binding sequence elements in gold. Microtubules, anionic phospholipids and some SH3 domains are thought to bind within the carboxy-terminal basic, proline-rich region. Amino-acid number is shown at bottom. PH, pleckstrin-homology domain.

domains, suggesting that it may serve as a critical link between signal transducing and endocytic pathways.

Over the past few years multiple families of GTPases have been identified, and many, if not all, of these enzymes are thought to perform regulatory roles — either as molecular switches like the trimeric G proteins and Ras, or as proof-reading enzymes like EF-Tu. Dynamin, first identified as a microtubule-binding protein, is a representative of a recently described family of high molecular weight GTPases (M_r 70–100K)². It is closely related to the product of the *shibire* gene in *Drosophila*^{3,4}, mutations in which block synaptic vesicle recycling in neurons and, more generally, the budding of vesicles from the plasma membrane^{5,6}. Mutant forms of mammalian dynamin have now also been shown to block receptor-mediated endocytosis when overexpressed in transfected cultured cells^{7,8}. So dynamin would seem to sit at a critical point in the control of endocytosis and synaptic vesicle recycling, but the detailed mechanism by which it functions in the cell remains to be worked out.

The present story began as part of a larger effort to characterize synaptic phosphoproteins. Upon depolarization of synaptosomes, a number of polypeptides were observed to become phosphorylated. However, a pair of polypeptides (of M_r about 100K) were specifically dephosphorylated (and were therefore termed

step of the dynamin GTPase pathway has yet to be defined, the stage in the pathway affected by phosphorylation is uncertain. However, it is tempting to speculate that dephosphorylation causes an increase in the lifetime of an 'activated' GTP-bound form of dynamin, with hydrolysis linked to a subsequent interaction with a downstream component of the endocytic pathway. According to this model, in the absence of electrical stimulation, when there would be little need for production of synaptic vesicles, GTP hydrolysis by dynamin would maintain the protein in an inactive state. Dephosphorylation would then couple GTP hydrolysis to synaptic vesicle formation.

The data of Robinson *et al.* suggest that protein kinase C phosphorylation occurs towards the carboxy terminus of dynamin. The carboxy-terminal 100 amino acids, which are unusually basic (pI = 12.5) and proline-rich (32 per cent)², have already been implicated in regulation. This region of dynamin binds microtubules, and its removal by papain cleavage abolishes the potent microtubule stimulation of the dynamin GTPase activity which has previously been observed^{10,11}. Furthermore, removal of the carboxy-terminal 188 amino acids (but not the carboxy-terminal 57 amino acids) from dominant mutant forms of dynamin reverses the inhibitory effect on endocytosis, suggesting that this domain is involved in mediating interactions with other components of the

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endocytic apparatus⁷. The composition of the dynamin carboxy terminus has also been noted to be consistent with phospholipid binding, and anionic phospholipids have been shown to stimulate the dynamin GTPase¹².

This region of the molecule also contains a number of sequences resembling the recently identified Abl-SH3 binding domain of 3BP1 (ref. 13), suggesting a possible interaction with SH3-containing proteins. In support of such an interaction, dynamin has now been identified as a prominent component of polypeptide fractions specifically bound to SH3 affinity columns (ref. 14; J. Schlessinger, A. Ullrich, personal communications). This is a particularly appealing development, considering the involvement of SH3 domains in signal transduction and that of dynamin in receptor-mediated endocytosis. Of additional interest, a region of dynamin upstream of the basic, proline-rich carboxy-terminal domain exhibits weak homology with pleckstrin, the major protein kinase C substrate of platelets^{15,16}. Similar domains have been identified in a variety of proteins known to play roles in signal transduction.

What all of these observations tell us about dynamin's mechanism of action is by no means clear. However, dynamin has emerged as a major control element in the endocytic and synaptic vesicle pathways, and it may well serve to link the endocytic pathway with signal-transducing systems. In this regard, dynamin should prove to be of profound interest in understanding a wide variety of growth regulatory and metabolic activities of the cell. □

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Ice sheets, wayward winds and sea change

Scott Lehman

LAST year, Bond and colleagues¹ showed that puzzling layers of detrital carbonate found in sediment cores resulted from occasions when great swarms of icebergs were discharged from the Laurentide ice sheet into the North Atlantic. It wasn't clear, though, how this fitted into the wider picture of climate change as documented in ice cores. On page 143 of this issue, Bond *et al.* begin to bring the two strands of evidence together².

There is no lack of new material to work with. Last September came the first report from one of two teams drilling on Greenland confirming that much of the period 8,000–40,000 years ago was marked by sudden 5–10°C switches in temperature over the ice sheet^{3,4}. In February came news from the other team that the switches were in fact jittery, embracing large oscillations in climate lasting in some cases less than 5 years⁵. And then in July came the further discovery that the past 8,000 years of relatively stable climate have been a geological oddity — the last time that there was as little ice on Earth as today (the Last Interglacial period), temperatures over Greenland varied even more wildly than during the glacial period, shifting by as much as 10–12 °C in just decades and remaining in place for as little as 70 years^{6,7}. Although climate modellers and geologists are racing to understand and test the implications of the new ice-core data, one thing already seems certain — the heat-carrying capacity of the Atlantic Ocean must somehow be involved in producing the sudden climate changes around Greenland.

Cycles

In one of the first studies to confirm this, Bond and co-workers² report that the past 90,000 years of temperature change on Greenland were indeed matched by changes in surface temperature of the northern Atlantic. And, as a by-product of their ocean-to-ice-sheet correlation exercise, Bond *et al.* have made an even more intriguing discovery. They find evidence in both the ocean and ice cores that the millennial oscillations in temperature known as Dansgaard-Oeschger events (see figure) appear to lump into longer-term 'bundles' in which the warm oscillations become successively cooler. In the ocean sediment cores these 'Bond cycles' culminate in evidence for brief but massive discharge of icebergs into the Atlantic. Following each of the iceberg discharge events, sea and air temperatures both rose sharply, marking the start of another Bond cycle. Fitting these events

into the somewhat more orderly march of Dansgaard-Oeschger cycles throws a new twist on an already confounding story.

Like the better-known climate oscillations at the end of the last glaciation, the Dansgaard-Oeschger events were probably caused by changes in strength of the ocean's conveyor-belt circulation system, a global-scale overturning marked by sinking of relatively cold and salty water in the Atlantic, generalized upwelling in the Indian and Pacific Oceans, and the return of warm salty surface water to the northern Atlantic around Africa and South America. The sinking of surface waters in the northern Atlantic is sustained by heat loss and salt enrichment due to evaporation, a process which both warms the air locally and, according to many oceanographers, propels the conveyor. As this sinking is density-driven, any process tending to decrease the salt content of northern Atlantic surface waters (increased precipitation, decreased evaporation, or ice-sheet, iceberg and sea-ice melting) would act to damp the conveyor and hence the flow of ocean heat towards the pole.

One prevailing theory holds that the conveyor undergoes millennial oscillations driven by the periodic build-up and decay of salt in North Atlantic surface waters in response to the changing balance between rates of vapour loss from the Atlantic to the Pacific, export of salt-laden deep water along the bottom of the Atlantic basin and the temperature-controlled melting of ice sheets⁸. To a degree, the results of Bond and co-workers support the 'salt oscillator' theory: they indicate that the poleward flow of warm surface waters changed in step with temperature oscillations seen in the ice cores. New geochemical results from the deep subtropical Atlantic are also compatible with the theory⁹. According to measurements of deep circulation proxies (the ¹³C/¹²C ratio of bottom-dwelling foraminifera), the flow of North Atlantic Deep Water varied on timescales similar to the surface ocean and atmospheric changes (see figure).

The layers of iceberg-derived sediments that now appear to mark the ends of the Bond cycles were first recognized in cores from the northeast Atlantic by Hartmut Heinrich¹⁰ and were later shown to occur along a broad swathe from Newfoundland to Portugal where prevailing currents brought bergs from North America into contact with warm subtropical water^{1,11}. Since known as Heinrich events, detailed radiocarbon dating shows that the layers were deposited nearly instantaneously

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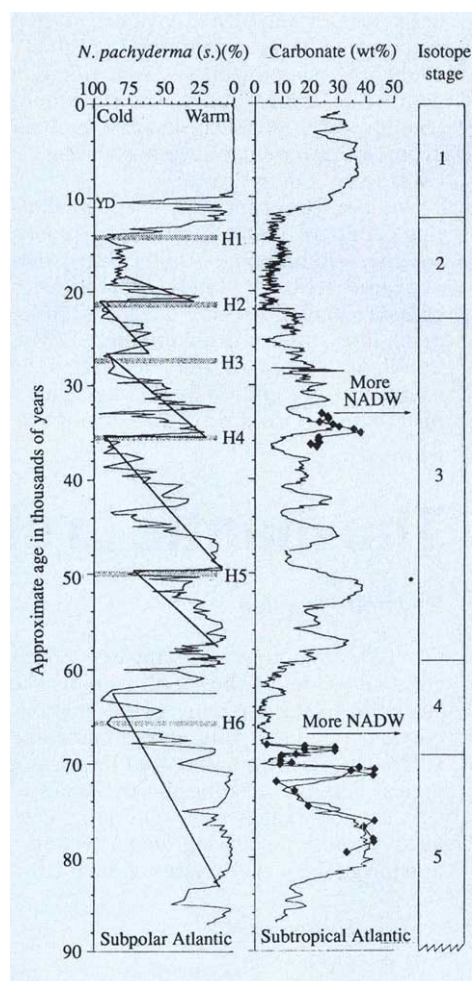
(within the few-hundred-year error of the dating technique), and indeed Bond and colleagues use these distinctive horizons as time-markers to correlate between ocean sediment cores.

Ice sheets

Although the evidence for the extent and timing of the Heinrich layers was assembled within just the past year-and-a-half, Doug MacAyeal has already formalized an ice-sheet model that may explain these events and possibly the longer-term characteristics of the Bond cycles (manuscripts submitted). MacAyeal's model gradually builds an ice sheet which, because it starts out predominantly frozen to its bed, is able to thicken without too much spreading. But the Earth's upward flux of geothermal heat acts continuously to bring more and more of the ice-sheet bed to the melting point. Eventually, the area of subglacial lubrication becomes so great that the ice sheet can no longer maintain its steep surface profile and collapses precipitously. In the process, ice streams away from the heart of the ice sheet, much of it to the sea through lubricated conduits such as Hudson's Strait. The newly thinned ice sheet then refreezes to the bed and the 'growth-purge cycle' begins anew. Using reasonable specifications of temperature and precipitation, the model produces a Heinrich (purge) event every 7,000 years, very similar to the geological record.

Whatever their cause, the breadth and rapid deposition of the Heinrich layers suggest that the fluxes of ice involved in their formation must have been enormous. According to MacAyeal's model, an amount about half the volume of today's Greenland ice sheet would have discharged in just a few hundred years. The release of such a large slug of melting ice into the northern Atlantic would undoubtedly affect the conveyor, producing brief bouts of cold even more severe than at the height of the last glaciation.

One of the most consistent results from ice-age simulations using atmospheric general circulation models is that the presence of a large ice sheet over North America acts to divert, cool and intensify westerly winds blowing across the Atlantic^{12,13}. MacAyeal speculates that the relatively slow thickening of the ice sheet gradually enhanced these effects, producing the long-term cooling trend seen within the Bond cycles. The sudden collapse of the ice sheet, on the other hand, may have permitted a rapid restoration of wind patterns similar to today's. Favouring evaporation over the Atlantic, these may have strengthened the conveyor to produce the warming seen immediately after the severe cold of the Heinrich events. Although difficult to prove, MacAyeal's idea may not be far off. Recent geochemical studies show that



Not only did temperatures at the surface of the subpolar North Atlantic (as indicated by the abundance of the cold-tolerant planktonic foraminifera *Neogloboquadrina pachyderma* (s.)) change on millennial timescales and in step with the 'Dansgaard-Oeschger' temperature cycles seen in Greenland ice cores, but the Dansgaard-Oeschger cycles themselves appear to bundle into longer-term, saw-toothed temperature cycles culminating in iceberg discharge (Heinrich) events, here labelled H1, H2 and so on². The surface ocean and air temperature oscillations were probably rooted in changes in the conveyor circulation — if so, they ought to have counterparts in the deep ocean. Recent studies of the carbon isotope content of bottom-dwelling foraminifera from 4.5 km depth in the subtropical Atlantic (solid symbols, range -0.5 to $+0.9$ ‰ PDB) indicate that this may be the case — production of North Atlantic Deep Water was significantly smaller during periods of harsher climate as indicated by smaller amounts of biogenic carbonate¹³. Although any attempt to correlate the subpolar and subtropical records would be subjective because of uncertainties in dating and the different climate sensitivities of *N. pachyderma* (s.) and carbonate percentages, the frequency and abruptness of changes in the two sediment cores are similar enough to suggest a connection. The next big questions are — what throttles the conveyor, how widely felt were the associated climate shifts, and how likely are they to occur in the future?

the flow of North Atlantic Deep Water was damped during the last Heinrich event (H1) but then increased to near modern levels just after (L. D. Keigwin and S. J. L., manuscript submitted).

There is now little doubt that the Pleistocene ice sheets were highly mobile features capable of exerting a considerable and sometimes abrupt influence on climate, but massive iceberg discharges cannot have been the dominant cause of the repeated weakening and strengthening of the conveyor 8,000–90,000 years ago. Ocean and air temperatures changed roughly every 1,000–2,000 years, whereas the Heinrich events appear to have occurred only every 7,000–12,000 years. And clearly large continental ice sheets could not have been players in the wild temperature variations of the last interglacial period. Although it remains to be proved that these variations had counterparts in the ocean, it seems very likely on the basis of evidence from the past 90,000 years. If so, it begs the question of what throttled the conveyor in a world largely free of ice.

Certainly, exploring the controls on conveyor circulation should remain a high priority. But from a practical point of view, an equally compelling question is how widespread the associated climate changes were. If similar changes were to

occur in the future, would they be restricted to the North Atlantic region? There are as yet no clear answers to this question, but there are growing indications that some of the climate shifts seen in the North Atlantic and over Greenland had equivalents as far afield as Florida, Chile and Antarctica. Grimm and co-workers¹⁴ recently reported evidence for large changes in vegetation in Florida that were apparently coeval with the past five Heinrich events. And in Chile, George Denton (personal communication) has found a series of moraines of equivalent age. Glacier advances within just a few drainages may not be climatically significant on their own, perhaps, but it seems

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RÉSUMÉ

Watered down

WATER conservation campaigns in response to the drought in California have certainly been effective; domestic usage in some urban areas has dropped by 28 per cent since 1986. R. A. Berk and colleagues were interested in exactly how this was achieved (*Clim. Change* **24**, 233–248; 1993). Changes in behaviour such as turning off the tap whilst brushing teeth are likely to be short-term, whereas technological fixes, such as low-flow shower heads or a brick in the cistern, should give long-term reductions. The research team approached the task with a healthy grain of scepticism, in which they were justified: of 632 Californians questioned on their conservation practices, 2 per cent claimed to recycle their light bulbs, 14 per cent apparently had an energy-saving television, and at least 10 per cent had heard of fictitious government campaigns to ban wearing fur, cutting Christmas trees or driving during smog alerts.

Inside information

ICEBERGS are not just a hazard for ships — they also cause headaches for those trying to plan oil and natural gas exploration or production in polar regions. The problem is that not much information is available about the movements of icebergs in the past, so it is hard to predict their likely paths in the future. S. Løset (*J. geophys. Res.* **98**, 10001–10012; 1993) now suggests that iceberg paths can be retraced from their internal temperature. He reports profiles of ice temperature versus depth obtained from icebergs in the Barents Sea, and shows that drilling deep enough (12 m into the ice) gives access to temperatures that are characteristic of the region from which the berg originally calved, and are unaffected by the temperature of the water through which it subsequently travelled.

Courses for horses

To test a horse's sporting performance, or see if it is under the weather, it can be more convenient to exercise the animal on a treadmill rather than on a track. The treadmill can be sloped to increase the workload of the running horse. But what angle best reproduces overground conditions when, among other differences, the horse will have a rider? E. Barrey *et al.* tackled this question, taking heart rate as the measured parameter (*Vet. Rec.* **133**, 183–185; 1993). They exercised seven horses at different speeds on a turf track, and compared heart rates for the same horses moving at various speeds on various slopes on a treadmill. From these analyses the optimal angle emerged as 3.5%, a result which was verified by re-runs on the track and on the treadmill with that slope. The 3.5% solution might, then, become a standard.

unlikely that repetitive advance in step with events in the Northern Hemisphere could have been fortuitous. And although damped in comparison to their northern counterparts, the Dansgaard–Oeschger events are apparent in several different ice cores from Antarctica⁷.

Wallace Broecker, the acknowledged ringleader of climate change studies, recently commented that what once appeared to be a stately procession of climate oscillations now seems to have been more of a “drunken lurch”. Undoubtedly, some of the inebriation he senses was brought on by the mechanical instabilities of ice sheets. But the implica-

tion from the Last Interglacial period that the conveyor may have flipped on and off in the absence of icebergs and meltwater helps to narrow the field of suspects. One of the more likely remaining culprits is the hydrological cycle, whose habits are still only poorly known. No doubt there will be heightened interest over the next few years in trying to document the strength and patterns of global vapour transport and their interactions with the conveyor. □

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SEISMOLOGY

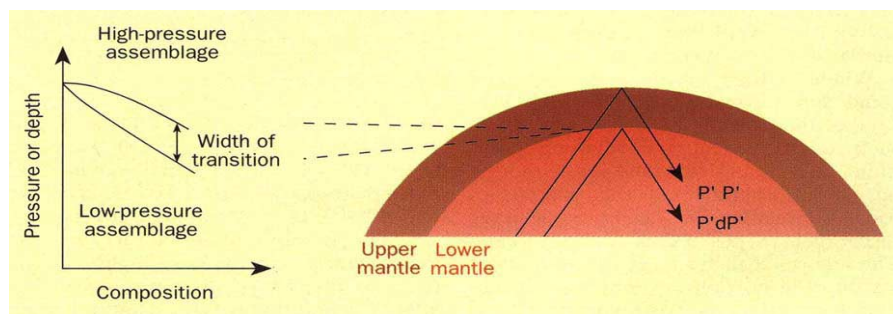
The mantle in sharper focus

Raymond Jeanloz

GLOBAL seismology is moving beyond the classical model of the Earth's onion-like structure, a static picture of the metallic core surrounded by the rocky mantle and crust. The new emphasis is on fine seismological details reflecting the dynamics of the interior; that is, the underlying convective motions driving plate tectonics and the geological processes of the Earth's

are ascribed to the olivine and pyroxene of the upper-mantle rock transforming to high-pressure spinel structures (γ -spinel and β -‘spineloid’ phases) at 14 GPa, which then transform to a mineral assemblage dominated by (Mg,Fe)SiO₃ perovskite at 23 GPa.

Like the Earth's ‘onion’ structure, a model based on thermodynamic equili-



The width of the coexistence loop for a high-pressure transformation among mineral phases (left) can be related to the amplitude of seismic reflections from the corresponding transition in the Earth's mantle (right). The magnitude of the impedance (velocity and density) contrast across the transition also determines the reflected amplitudes. P'P' and P'dP' are two different seismic ray paths.

crust. Such details include the horizontal variations (a few per cent in magnitude) in wave velocities determined from seismic tomography, and the kilometre-sized undulations of the interfaces between layers. New studies of interface reflectivities may also tell us much about the Earth's internal dynamics, as Benz and Vidale show on page 147 of this issue¹.

The traditional interpretation of mantle structure, the subdivision into upper mantle, transition zone and lower mantle, is in terms of equilibrium transformations among mineral phases^{2,3}. The discontinuous changes in seismic velocities at 400–410 and 650–670 km depth, defining the top and bottom of the transition zone,

brum is essentially static in nature, yet has the advantage of making detailed predictions of what should be observed at depth. Consequently, deviations from the phase-equilibrium predictions can provide insight into the dynamics of the mantle (alternatively, such deviations may simply be showing that our models of the interior are wrong). A specific prediction is that phase transitions — and hence the corresponding seismic discontinuities — should occur over a finite depth interval, in general. The width of each transition or discontinuity is given by the pressure interval across the coexistence loop in the equilibrium phase diagram (see figure). Thus, seismic reflections become

particularly interesting in offering a combined measurement of the sharpness and impedance contrast (velocity and density jump) of the discontinuities.

Previous work shows that the reflectivities of the mid-mantle discontinuities can be large: about 5–10 per cent, implying discontinuities less than about 2–5 km wide⁴. But these observations have been made in only a few locations and have not been particularly reproducible; reflections from 400–410 and 660–670 km depth are not always present, and reflections from other depths sometimes appear. One possible interpretation has been that strong reflections are only observed under special circumstances, for example where local curvature of the discontinuity provides optimal focusing of the reflected waves^{4,5}. If so, the strong reflections could be considered anomalous, with the lack of strong reflections (and therefore transitions more than 10–20 km wide) being the norm.

It is here that Benz and Vidale's contribution stands out. Using an array of over 700 seismometers, they are able to document the mid-mantle reflections with unprecedented clarity. The array offers unique evidence for lateral coherence of the reflecting discontinuities over distances of hundreds of kilometres. They find strong reflections from the 660-km discontinuity defining the top of the lower mantle, clear but more intermittent reflections from the 410-km discontinuity at the top of the transition zone, and no evidence for any other discrete structure within the transition zone. Where observed, the two discontinuities must therefore be strong and sharp, less than 2 km (about a quarter wavelength) in thickness.

At face value, Benz and Vidale's result is incompatible with the details of the equilibrium phase diagrams^{4,6–8}, implying that there is more to the transition zone than simple mineral transformations. Unlike the authors, however, I would say that the 660 rather than the 410-km discontinuity causes the most trouble. The reflections from the latter can probably be explained in terms of a combined pyroxene and olivine assemblage of the upper mantle transforming to the spinel assemblage of the transition zone⁴. This interpretation is compatible with the intermittent character of the 410-km reflection, the coupled transformations of pyroxene and olivine being barely adequate to satisfy the observations. Also, new elasticity measurements suggest a larger impedance contrast across the olivine–spinel transition than previously thought⁹.

In my view, it is the strong, coherent reflections from 660 km depth that are difficult to explain through phase transitions alone. Despite Ito and co-workers' proposal that the spinel to perovskite transition is narrow⁶, the new seismic observations require a transition width far

smaller than can be resolved in the high-pressure experiments. Yet even for an infinitely narrow transition, a larger impedance contrast is required at 660 km depth than is justified by existing elasticity measurements^{4,10,11}.

It is for these reasons that the seismic reflections seem to require a dynamic (rather than equilibrium thermodynamic) explanation, especially for the 660-km discontinuity. One possibility is that mantle convection is intrinsically layered, because of a difference in bulk composition between the upper and lower mantle^{10,11}. Whether or not this is the case, the recognition that mantle convection is profoundly influenced by the occurrence of phase transitions^{12,13} suggests that the pattern of flow in the transition zone could be perturbed enough to create reflective discontinuities. Similar ideas are now being considered for the seismic properties of the Moho, the interface between crust and mantle: thin lithological or textural layering induced by large-scale lateral shearing in the transition zone might produce coherent reflections that

are strong enough to explain the observations. Whatever their ultimate explanation, the seismological data will be examined for all they can tell us of the dynamics of the Earth's mantle. □

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MAJOR HISTOCOMPATIBILITY COMPLEX

The evolutionary angle

Julie Clayton and Henry Gee

THE long-standing job of immunologists is to work out how the immune system fights infection. But one of the components of that system — the major histocompatibility complex (MHC) — is turning out to be a rich source of information about animal evolution, and a band of enthusiasts recently got together* to see what can be surmised from the latest work in the area.

Proteins encoded by the MHC transport peptide fragments of foreign antigen from intracellular compartments and display them at the cell surface to T cells of the immune system. MHC molecules are of two types — class I (which pick up peptides derived from intracellular biosynthesis) and class II (which collect peptides derived from proteins taken in by endocytosis from outside the cell). The basic design of these molecules occurs in a multitude of polymorphic variations, each of which favours binding of certain clusters of peptide sequences. But each MHC locus contains no more than two polymorphic alleles in an individual. Different individuals are thus differently protected.

Relationships

The general outlines of the MHC can be traced back to the earliest vertebrates and the unmatched polymorphism of the genes concerned can be used to reveal

evolutionary relationships through time. A great deal of that polymorphism has been revealed, but it is clear that much more awaits discovery. Thus the finding that the highly divergent class I HLA (human leukocyte antigen)-A*8001 allele, and the class II allele HLA-DR3 Drw18, seem to be unique to African Americans is no surprise given that most previous studies had been done on Caucasians (P. Parham, Stanford University; H. Erlich, Roche, Alameda). These alleles could be but the tip of the iceberg, a reflection of a large number that may be typical to Africa and not found in populations that migrated to the other parts of the world.

Nevertheless, HLA alleles have already been used to trace certain human migrations. For example, populations from Siberia to Amazonia each have their peculiar variants of HLA-B*4002, but *4002 itself is also present as a thread linking people as diverse as Japanese businessmen and Amazonian hunters to the long-dispersed population that first crossed into Alaska across the Bering Straits around the end of the last period of glaciation.

Interestingly, new HLA alleles appear to have arisen in the Indian peoples of South America^{1,2} (Parham; D. Watkins, University of Wisconsin). Much of this diversity is concentrated in the peptide-binding region of HLA-B, hence the en-

*Evolution of the Major Histocompatibility Complex: Causes and Effects, King's College, Cambridge, and the AFRC Babraham Institute, Babraham, UK, 10–14 July 1993.

ting idea that these new alleles arose in response to selection pressures from disease encountered by Indian populations as they migrated southwards into the tropics. Disease resistance is also the most likely explanation for the significant excess of MHC heterozygotes among the Havasupai, a small tribe of about 600 people living in the splendid isolation of a side-gorge off the Grand Canyon (P. Hedrick, Arizona State University). Heterozygotes at the MHC seem to have a selective advantage over homozygotes because they can present more distinct peptide sequences to the immune system.

The identification of the HLA-B locus as particularly responsive to selection pressure is not confined to the Americas, or to people. Thus whereas the human class I HLA-A alleles A3/A1/A11 have close relatives in the common and pygmy chimpanzees *Pan troglodytes* and *P. paniscus*, there are considerable differences among class I HLA-B alleles in these species, particularly in the peptide-binding region.

What might be the mechanisms of production of new allelic variants in the human MHC? Gene conversion (exchanges of short segments of nucleotides between closely related alleles), it seems, is at least part of the answer. The human class I allele HLA-B76, for example, found in Thais, has probably arisen by the substitution of just two amino acids at positions 166 and 167 (Parham). Most presumed conversions in MHC class I appear to involve the region coding for the peptide-binding site, but it is not known whether this is due to specificity in the conversion mechanism or to subsequent selection by disease.

Of the human class II alleles, diversification has occurred at all three of the main loci, DR, DP and DQ. For unknown reasons, different DP alleles seem to be of strikingly high frequency in different ethnic groups: the HLA-DP0501 allele, for example, is most prevalent in Japanese and south-east Asian communities (A. Begovitch, Roche, Alameda). So DP variations hold promise as ethnic or population-specific markers.

MHC alleles can be grouped according to sequence similarity into allelic lineages, which presumably reveal a common ancestry. Thus alleles of class II DRB1 and DRB6 genes are shared between humans, gorillas and chimpanzees. But there are new allelic variants in each species, showing that the DRB1 and DRB6 lineages are more than 25 million years old (R. Bontrop, University Hospital, Leiden). Furthermore, it seems that these molecules are functionally conserved in the peptide-binding region — antigen-presenting cells taken from rhesus macaques, for example, can present a peptide of the 65K heat-shock protein of *Mycobacterium leprae* to human T cells,

where both species share MHC DRB1*03 lineage molecules.

Going further back in time, there is the issue of whether New World monkeys (platyrrhines) and Old World simians (catarrhines) have a common ancestry or diverged independently from prosimian primates. As with humans, monkey MHC alleles generally differ at peptide-binding sites, but platyrrhine MHC genes have peculiar sequence 'motifs' in class II genes DRB1, 3 and 5 (W. Mayer, Max-Planck-Institut, Tübingen). It seems clear that these genes evolved more than 37 million years ago, probably before the divergence of platyrrhines and catarrhines (although fossil evidence tends to put divergences between primate groups even further back³).

Pseudogenes

Analysis of platyrrhine MHC genes (which are mostly non-functional pseudogenes) also reveals how new genes can be created from the ruins of the old (Mayer). The pseudogene DRB6, for example, is no longer active in platyrrhines, but is transcribed in catarrhines and humans. It seems that a functional promoter resembling that of mouse mammary tumour virus is responsible for gene expression, suggesting that a retroviral insertion occurred in the ancestral DRB6 gene more than 23 million years ago.

The advent of the prosimians lies even further back in time (more than 85 million years ago). Human-like class II DRB-1, 3 and 6 genes are found in extant examples of this group, such as bushbabies and lemurs, and phylogenetic trees show similarities and consistency with primates more generally (F. Figueroa, Max-Planck-Institut, Tübingen).

The persistence of alleles and allelic lineages through speciation events is not only evident in mammals. Analysis of a few of the hundreds of species of cichlid fishes in Africa's Lake Malawi and tributary rivers has shown that the founder population must have been highly polymorphic, and much of its allelic diversity has been passed down to the descendant species, all of which have arisen within the past two million years (Jan Klein, Max-Planck-Institut, Tübingen). So far, there is no evidence for the evolution of clusters of new species-specific alleles, such as have arisen in the class I HLA-B genes of the South American Indians. The inference to be made here is that the lake has provided a relatively stable environment, at least in terms of disease, compared to that encountered by migrating peoples⁴. □

Julie Clayton and Henry Gee are assistant editors at Nature.

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Sparkling water

LAST week Daedalus decided that seals and dolphins were protected from the 'bends' by a bubble-inhibitor in their blood. He planned to identify and synthesize this material, and use it to load water with a vast amount of air which nonetheless could not bubble out. A swimming bath doped with DREADCO's 'Nobub' could hold so much oxygen that swimmers could breathe the water.

Daedalus now wants to try this trick on its obvious beneficiaries — fish. They are seriously restricted by the low solubility of oxygen in water. An aquarium supersaturated with oxygen and stabilized with Nobub would, for the first time, give them a copious oxygen supply. They should become much more active, and grow with wonderful rapidity. By breeding fish selectively in such an aquarium, DREADCO's biologists hope to produce strains adapted to exploit this novel environment: energetic, fast-growing and highly nutritious. The ultimate goal is a supersaturated fish farm with vastly improved yields.

The farm, of course, will generate a lot of excreta and general water pollution. Daedalus argues that this will be rapidly biodegraded in the oxygen-rich water. Similarly, he reckons that Nobub could help to clean up polluted rivers and lakes. A pressurization-unit could pump many atmospheres of oxygen into a Nobub-treated river, where it would accelerate natural purification. The most extreme biological oxygen demands would be effortlessly met. Industry and sewage and refuse-disposal services could go back to their old, cheap, simple policy of just dumping their wastes in the nearest river, while trusting Nobub and compressed air to clean up the mess. Nobub itself, having evolved to stabilize the blood-oxygen of aquatic mammals, will of course resist oxidation.

Small-scale uses for Nobub could also appear. Many housewives believe in the cleaning ability of detergent foam. DREADCO's Nobub detergent seems foam-free, despite being supersaturated with oxygen. But when a special Nobub-antagonist is added, the resulting foamy blast dislodges the most obdurate stains. Daedalus also began to devise a Nobub-doped beer, which would not foam crazily out of the freshly opened can or bottle. It would only seem fizz-free, however. As its content of Nobub was digested in the drinker's stomach, it would release its concealed gas, blasting him with almost terminal flatulence. So Daedalus now plans to counter fizzy beer by serving it in glasses lined with a Nobub-antagonist, to get rid of the wretched gas before it enters the drinker.

David Jones

HIV-1 inhibition by a peptide

SIR — Studies on interactions between the domains of peptides involved in assembly of some enveloped RNA viruses suggest that peptides from virus envelope glycoproteins have antiviral activity¹. A peptide from HIV-1 gp41 (DP-107: NNLLRAIEAQHLLQLTVWGKIQ-LQARILAVERYLKDQ) has antiviral activity², for example. We have found that a peptide from another gp41 region (amino-acid residues 637–666: EWDREINNYTSLIHLIESQNQQE-KNEQEGGC) also strongly inhibits HIV-1-IIIB replication. The 50% effective doses (ED₅₀) for inhibition of gag p24 nucleocapsid protein production, the cytopathogenic effect (CPE) and cell fusion were 101, 142 and 156 nM, respectively (*a* in the figure). HIV-1 infection was completely inhibited at concentra-

tions of peptide greater than 2 μ M.

Peptide (637–666) inhibits infection by other HIV-1 strains: MN; RF; SF2; V32 (a IIIB variant resistant to neutralization by antibodies directed against the IIIB V3 loop of gp120, ref. 3); and HIV-1 derived from clinical isolates differing in sensitivity to AZT⁴ (*b* in the figure). These results suggest that the peptide has antiviral activity against both homologous and heterologous HIV-1.

We synthesized the peptide (637–666) with a GGC linker at its carboxy terminus for convenience of peptide coupling to carriers or labelling. Mass spectrometric data (provided by B. T. Chait and U. Mirza, Rockefeller University) demonstrated that peptide (637–666) is dimerized. The monomeric form of (637–666), prepared by reduction with 1% 2-mercaptoethanol and alkylation with 3% iodoacetamide, has ED₅₀ values for inhibition of p24 production, CPE and cell fusion, respectively, 3 to 4 times higher than those corresponding to the dimeric (637–666) peptide.

The peptide (637–666) has no detectable cytotoxicity for various cell lines tested or for peripheral blood lymphocytes and monocytes at a concentration of 816 μ M, about 8,000 times higher than the ED₅₀ values. Fresh sera from HIV-1-negative individuals did not interfere with the anti-HIV-1 activity of the peptide. The presence of antibodies recognizing this peptide in sera of HIV-1-infected indi-

viduals is not expected to interfere with antiviral activity, as the effective peptide concentration greatly exceeds the concentration of such antibodies. Furthermore, unconjugated peptide is not expected to elicit antibodies, particularly when administered without an adjuvant.

The mechanism by which the peptide (637–666) inhibits HIV-1 infection is under investigation. The peptide did not inhibit binding of HIV-1 to target cells but selectively bound to the virus fusion domain, suggesting that it inhibits HIV-1 infection by preventing the interaction between the fusogenic domain of gp41 and relevant cell membrane constituents, thereby blocking fusion of HIV-1 with cells or of infected cells with uninfected cells.

In summary, the peptide (637–666) has antiviral activity against both homologous and heterologous HIV-1 isolates and has no detectable cytotoxicity. It thus offers another approach to attempts to devise treatments for AIDS.

Shibo Jiang

Kang Lin

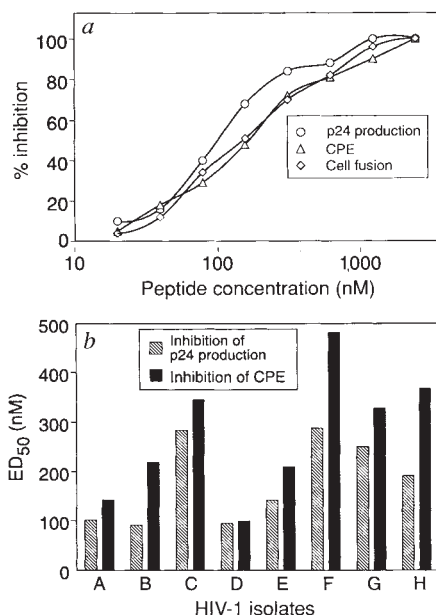
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Inhibition by peptide (637–666) of infection by *a*, HIV-1-IIIB and *b*, by different isolates: IIIB (A); MN (B); RF (C); SF2 (D); V32 (E); and HIV-1 sensitive (F), partially resistant (G), and resistant to AZT (H). Infection of MT-2 cells by HIV-1-IIIB or by other HIV-1 isolates (multiplicity of infection 0.0045) in the presence or absence of the peptide (637–666) was detected by ELISA for p24 production and a colorimetric assay for CPE as previously described⁵. Cell fusion was measured as follows: HIV-1-IIIB infected H9 cells were labelled by 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Inc.) according to the manufacturer's instructions, and mixed with uninfected MT-2 cells. After incubation, the fused and unfused BCECF-labelled H9/HIV-1-IIIB cells were counted under an inverted fluorescence microscope and the per cent inhibition of cell fusion was calculated. HIV-1 V32 was provided by P. L. Nara; all other HIV-1 isolates were obtained from the NIH AIDS Research & Reference Reagent Program.

Nested fullerene-like structures

SIR — Hollow carbon structures consisting of nested graphitic shells have been reported for various geometries^{1–3}, and the filling of these structures with metal compounds has also been described^{4–6}. Our discovery⁷ that similar hollow structures can be observed for the layered semiconductor tungsten disulphide leads one to expect that these structures might be formed from other layered materials. We have now found that molybdenum disulphide will also form such concentric structures, and that they too can be filled with metal compounds.

We deposited molybdenum films of 20 nm thickness onto quartz substrates, oxidized them at 500–600 °C in open air and fired them at 850–1,050 °C in a stream of H₂S and N₂/H₂ mixture. We peeled the MoS₂ films obtained off the substrate and examined them in the transmission electron microscope.

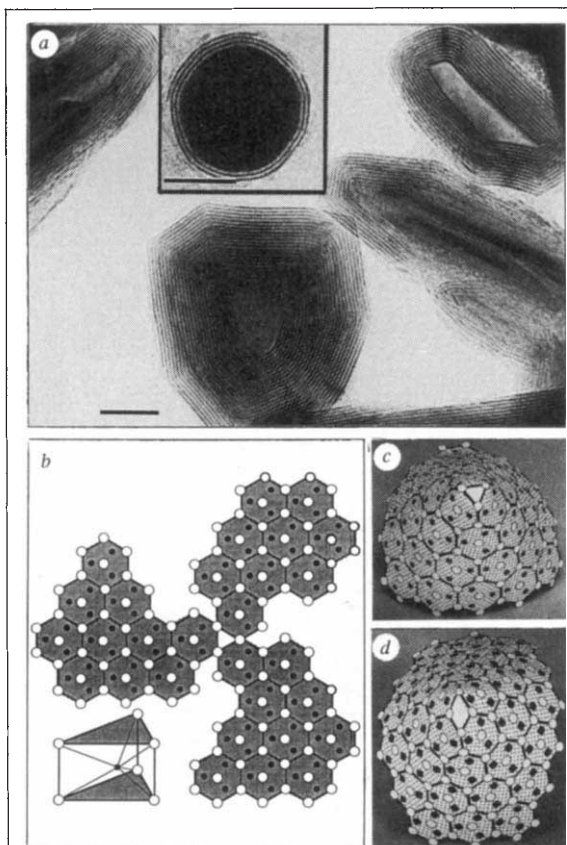
The figure (*a*) shows the lattice image of a few nested shells of MoS₂. The nested shells seem to be hollow in most cases. We also saw, albeit rarely, 'stuffed' structures, composed of molybdenum-dense core

(confirmed by local energy dispersive X-ray analysis) wrapped with a few MoS₂ layers (inset in figure). X-ray photoelectron spectroscopy analysis of the films reveals a Mo:S atomic ratio of 1:2. This result indicates that the oxide serves as a precursor for the generation of a nested shell, rather than being a part of it.

We observed various apex angles, predominantly 120° and larger, but often of 60, 75 and 90°. Tilting about different axes shows that these angles are not necessarily symmetrical.

Some of the apex angles can be deduced from the MoS₂ layer structure. The figure (*b*, *c*, *d*) demonstrates how an apex can be created by folding and joining either three or four hexagons around a triangle or rhombus, respectively. Further growth of the cone may be explained by a model described by Kroto⁸ for carbon fullerenes. Only one point defect — a molybdenum vacancy — is required for initiating this process. The generation of the 'stuffed' shells can be ascribed to a wrapping mechanism of spiral growth⁹.

Structural transformations in the films



a, Electron micrograph of a region containing a few nested polyhedral shells. The interlayer distance in the different faces of the polyhedra is 0.62 nm, which coincides with the $c/2$ lattice parameter of 2H-MoS₂ polytype. A 'stuffed' Mo core with an incomplete MoS₂ shell is shown in the inset. Scale bars, 10 nm. b, Two-dimensional drawing of the Mo-S-Mo basic triple layer and a view of the elemental trigonal prism of 2H-MoS₂ lattice. Open circles, sulphur; closed circles, molybdenum. c, Three-dimensional drawing of a conical apex of 60° angle formed by folding and joining three hexagons around a triangle (as shown in b). d, Three-dimensional drawing of a conical asymmetric apex formed by folding and joining four hexagons around a rhombus, with 90° along the short axis and 75° along the long axis.

were studied by X-ray diffraction and absorption spectra. At 850 °C (30 min annealing time) most of the integrated intensity of the X-ray spectrum is confined to a wide peak of the amorphous material, and the (002) peak of the crystalline MoS₂ is very weak. The absorption spectrum does not show a particular structure. At 1,050 °C, on the other hand, a strong (002) peak of the crystalline 2H-MoS₂ phase predominates and clear excitonic

peaks are observed at 680 and 630 nm (A and B excitons). Similar transformation of the diffraction peaks occurs when the annealing time (at 950 °C) increases from 5 min to 1 h. These results show that the amorphous material gradually transforms into a crystalline phase on annealing in H₂S atmosphere. The higher the temperature, the shorter is the time required for this transformation to occur.

At early stages of the annealing one observes in the electron microscope partially crystallized clusters, embedded in amorphous matrix. Tilting the sample at various angles inside the microscope reveals the incomplete structure of the nested shell. This stage is reminiscent of the closure of carbon polyhedra^{8,10,11}. Annealing the films at 850 °C for 30 min or more yields a mixture of phases, containing round-shaped globules of about 100 nm or larger, and many hollow shells less than 20 nm in diameter.

The synthesis of MoS₂ from MoO₃ and sulphur (or H₂S) is well established¹². The reaction leading from MoS₃ to MoS₂ is also known. A series of phase transformations occurs which can be represented by the sequence a:Mo → a:MoO₃ → a:MoS₃ → c:2H-MoS₂ (where 'a' means amorphous and 'c'

crystalline). The nested-shell phase is an intermediate stage between the last two phases.

This and previous⁷ work shows that closed polyhedra are a common phenomenon in materials with layered structure. One may now predict the discovery of nested shells in other layered materials: for example, our preliminary experiments confirm their presence in Mo(W) diselenides.

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Herbicides and transgenic rape

SIR — We congratulate M. Crawley *et al.* (*Nature* **363**, 620–623; 1993) on providing the first extensive study on the performance of transgenic oilseed rape in natural habitats. It is to be hoped that this study will be one of many which aim to provide data rather than rhetoric on an emotive subject in which opinions are polarized.

Unfortunately, given the paucity of information currently available on the subject of 'invasiveness', there is also a danger of over-interpreting a limited amount of data. Crawley *et al.* state that their results suggest that "herbicide-tolerant oilseed rape poses no greater threat to the environment than the conventional crop". To draw such a general conclusion may not be justified as Crawley *et al.* deal with only one herbicide — glufosinate. Extrapolating these results to other herbicides is not necessarily valid as the modes of action and mechanisms of resistance of various herbicides vary greatly.

In relation to glufosinate in particular, feral oilseed rape populations are regularly encountered on waste ground and in the margins of agricultural fields. Glufosinate is one of the herbicides approved for weed control in such locations. Farmers or local authorities attempting to deal with a spectrum of weed species, including a feral rape population which they did not know to be glufosinate-resistant, might well apply this herbicide as a control strategy. The lack of effectiveness of this treatment on the rape, together with the removal of competition by the other species present, would give the feral population a marked selective advantage.

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Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

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Bangs replace whimpers

Gordon L. Herries Davies

The New Catastrophism: The Importance of the Rare Event in Geological History. By Derek Ager. Cambridge University Press: 1993. Pp. 231. £22.95, \$34.95.

"FASCIST!" Among street politicians that is the ultimate abuse shouted as a prelude to yet more violent leftist action. "Catastrophist!" In my early days that was the ultimate insult to be hurled at an Earth scientist who seemed to be straying outside the prevailing dogma of strict uniformitarianism. We of course accepted the reality of occasional terrestrial catastrophes. I had relished my grandmother's teenage memories of the glorious English sunsets that had followed the eruption of Krakatoa in 1883 and I had just completed the Hebridean fieldwork for my undergraduate dissertation when disaster struck at Lynmouth on 15 August 1952. But all such events were seen just as natural aberrations of only temporary significance.

We preferred to believe that what was important in geohistory was nature's long-term, gradualistic processes. The Grand Canyon was entirely a result of the Colorado River's daily removal of x soil-grains over millions of years. Sedimentary strata formed in a marine environment were interpreted as the little-by-little accumulation of particles raining down on the sea bed over aeons of time. What seemed to matter was the ceaseless tick-tock of the natural clock. That the sound of the ticking was occasionally drowned by a ringing of the clock's alarm seemed immaterial.

Now all is changed. We are rewriting geohistory. Where once we saw a smooth conveyor belt, we now see a stepped escalator. Upon that escalator the treads are long periods of relative quiescence when little happens. The risers are episodes of relatively sudden change when the landscape and its inhabitants are translated into some fresh state. Even the most staid of modern geologists are invoking sedimentary surges, explosive phases of organic evolution, volcanic blackouts, continental collisions and terrifying meteoroid impacts. We live in an age of neocatastrophism.

Catastrophism was originally born during the seventeenth century in an attempt to cram some inkling of the complexity of geohistory into the pint pot represented by biblical chronology. I suspect that the catastrophists of that distant age might well find themselves far more at home in our modern departments of geology than

would their uniformitarian successors in the nineteenth century.

The late Derek Ager needs no introduction to a geological audience. For nongeologists I simply observe that he was a distinguished Anglo-Welsh stratigrapher. His stimulating book *The Nature of the Stratigraphical Record*, first published 20 years ago, has been widely read in two editions (a third is in the press) and

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Earth in transition — Arizona's meteor crater, which is about 200 metres deep and 800 metres across, serves as "irrefragable evidence of the importance of that sudden but rare event".

in seven continents (some thoughtful geologist *must* have taken a copy to the Antarctic). His new work shares with its predecessor the qualities of originality, readability and seminality. I thoroughly recommend it. On every page we encounter as our guide an idiosyncratic (the word is intended as a compliment), hammer-carrying field geologist of a type increasingly rare in the modern world. He tells us that his "petrological hero" is the late H. H. Read and I am reminded that at the conclusion of his final field-excursion to County Donegal, Read left his field boots atop a dry-stone wall "as a gift to posterity". Ager was another great character of the same kind.

The nature of the book is revealed best by its subtitle rather than by its title. This is no broad, airy-fairy overview of neocatastrophism. It is, rather, a feet-on-the-ground, sweat-in-the-eye encounter with an abundance of actual geological sites. Some of these sites have served to convince Ager that the rocks of our globe contain irrefragable evidence of the importance of that sudden but rare event. As

a vastly experienced field-observer (at the time of his writing he had plied his hammer to the face of 57 countries) he takes us from a Norwegian landslip to pillow-lavas in New Zealand and from Arizona's meteor crater to the flysch of Hokkaido. And everywhere the message is the same. We are on an escalator, not a conveyor belt.

On page xvi, I found a sentence that reverberates inside my head: "The geological record is constantly lying to us". Is that statement true? Are rocks not utterly passive in their stony silence? Surely what we know as geohistory originates not within rocks but within the minds of their human observers. As a creation of the human intellect, our geohistories may owe more than is commonly supposed to processes acting within our own cerebra. One of Ager's heroes is Cuvier. Was he inclined towards catastrophic interpretations of the stratigraphical column because he had witnessed the toppling of the *ancien régime*? Did Lyell, that high priest of uniformitarianism, exclude catastrophe from his geological system out of self-interest? For an affluent member of the British establishment, social upheaval could spell only personal disaster. Did Lyell therefore place his taboo upon all manner of speculation invoking revolutionary turmoil? Have today's geologists invented and espoused neocatastrophism because they have lost the self-confidence enjoyed by our forebears? Do we believe in bygone catastrophes because we hold our very existence to be threatened by a multitude of

potential catastrophes ranging from mass starvation to renewed glaciation?

Ager devotes several pages to the origin of the channelled scablands of Washington State. To my regret I never met the geologist who originated the now widely accepted view that those remarkable landscapes resulted from catastrophic late-glacial flooding. Such confidence did that geologist have in his theory that he took wry satisfaction in being branded a catastrophist by those benighted uniformitarians who dismissed his startling interpretation as absurd. But what really did annoy him was the critics who misspelled his name. Now Ager has fallen into the trap laid for us so long ago by that American geologist's parents. The error is in two senses just a small point. The American geologist's name was J Harlen Bretz. The J was a given name and not an abbreviation. □

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Battlefield archaeology under fire

Shepard Krech III

Archaeology, History, and Custer's Last Battle: The Little Big Horn Reexamined. By Richard Allan Fox Jr. *University of Oklahoma Press*: 1993. Pp. 411. \$29.95.

IN 1983, a prairie fire ravaged over 600 acres at Custer Battlefield National Monument (since renamed Little Bighorn Battlefield National Monument, in belated recognition of Indian participation on both sides). This Montana site commemorates what may be the single most debated battle in American history: Cus-

The general details of the events preceding Custer's Last Stand and the battle itself are well known: the duplicitous United States, which stopped neither its own army nor gold-rushers from marching into the Black Hills in violation of the Treaty of 1868 guaranteeing the security of these lands; the Sioux and Northern Cheyenne, fed-up, declared "hostile" because they chose not to report to reservations, but to gather in the valley of the Little Bighorn for ceremony, renaissance and bison in the largest gathering ever on the plains; Custer, a vainglorious careerist and inept strategist; and the chaotic battle of 25–26 June 1876, which was a disaster for the US Army's 7th Cavalry and a pyrrhic victory for the Sioux and their allies, who were soon defeated for good and confined to reservations.

Mary Evans

None of this is altered by archaeological evidence. Nevertheless, Fox reiterates earlier published conclusions from his team's archaeological investigations, including the impressive confirmation of Indian sources that Indians used a great variety of guns in the battle. He then turns to the more weighty arena of battle tactics and behaviour under fire, which is where he intends this book to make its contribution. Fox is most interested in the relationship between expected (or demanded) and actual behaviour, in group

stability or disintegration under stress and in the will to fight — in short, in models of combat behaviour — and by weighing in with spent bullets and cartridge cases (the bulk of the archaeological evidence) he is able to suggest alternative explanations for some of the events of this battle. As some documentary sources stress, the behaviour of Custer and his men was marked by disintegration and bunching around the leader; not surprisingly, the image of Custer blazing away in the final throes of battle on the hill where he was said to have died, which became so popular in text and painting — one of the best known being Edgar Paxson's monumental work, *Custer's Last Stand* (1899) — is undermined by the archaeological evidence, as it has been by accounts based on documentary evidence alone.

One interpretive problem facing Fox concerns the nature of the Battle of the Little Bighorn and the debate over it: the battle was made up of many skirmishes

which have been extensively discussed. Because he favours model-building over the documentary record, Fox never provides an adequate context for distinguishing authoritative or authentic accounts of battle from those that are in some way lacking. In fact, it is often difficult to discern in Fox's narrative the orthodox explanation of troop behaviour at any one time. A summary of how his archaeology supports or refutes documentary evidence does not really help, because of the extraordinary confusion of the battle, the sheer number of accounts from individual participants and the instant mythicization of Custer and the savaging of his enemies. The result is that one does not quite understand the extent to which archaeology upsets a particular era's conventional wisdom.

Fox is far from unaware of these problems, and in fact speaks throughout the book to what he sees as the difference between anthropological and historical explanation. When he says that he is an archaeologist not a historian, he means he is interested in process and regularity rather than description. He clearly favours deductive reasoning, but by leading with archaeological evidence, not with competing versions of battle events, Fox never quite manages to provide the context for source criticism or archaeological clarification. Many historians would not only rightly object to Fox's distinction between their discipline and anthropology, but they tend also to have a far greater insight into which among competing sources are to be given credence. The structure of *Archaeology, History, and Custer's Last Battle*, forced by Fox's faith in the primacy of archaeological evidence, precludes what might have been a more logical route: re-examination and evaluation of the extensive battle accounts — eyewitness or not, recorded immediately or long after the fact, Indian or White, and so on — embedded firmly in the context of their cultural and historical reproduction, then measured in the light of fragmentary archaeological remains.

These remarks are not intended to be hostile to the importance of material remains to writing history, but as a reminder that because interpretation of archaeological evidence and documentary sources is equally perspectival, one must be wary of placing too much faith in (so-called) scientific approaches to objects alone. This book will certainly give the Custer industry plenty to chew on, and it will also make battlefield archaeologists anywhere think twice about their own analyses; but despite Fox's protestations, historical archaeology performs best in supporting historiography. □

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Inept strategist — Custer at Little Bighorn.

ter's Last Stand, in which the Sioux decisively defeated General George Armstrong Custer, killing him and more than 260 men under his command. For battlefield archaeologists, fire was opportunity. Before it, they were on the sidelines of the so-called Custer Industry — the interminable argument over the historiography of the Last Stand. But after the burn, archaeologists quickly unpacked their metal detectors, trowels and shovels, surveyed the site, excavated troop remains, discovered more than 1,000 spent bullets, cartridge cases and other artefacts, and stepped onto the playing field.

Unlike many other archaeologists, they published the results of their research promptly; Richard Fox's *Archaeology, History, and Custer's Last Battle* is the third major book to come from their exertions. The most ambitious and deeply and elegantly — if redundantly — reasoned of all, it reveals historical archaeology's strengths as well as limits.

New in paperback

Complexity: Life on the Edge of Chaos by Roger Lewin. Phoenix, £6.99. Reviewed in *Nature* **361**, 507 (1993).

The Evolution of *Homo erectus*: Comparative Anatomical Studies of an Extinct Human Species by G. Philip Rightmire. Cambridge University Press, £16.95, \$24.95. Reviewed by M. Day in *Nature* **348**, 688 (1990).

Mammoths, Mastodonts, and Elephants: Biology, Behavior, and the Fossil Record by Gary Haynes. CUP, £16.95, \$27.95.

The Two Cultures by C. P. Snow. The physicist and novelist's influential 1959 Rede lecture, now issued with his successor piece, *A Second Look*, in CUP's Canto series, £5.95, \$9.95.

Killer Bees: The Africanized Honey Bee in the Americas by Mark L. Winston. Harvard University Press, \$10.95, £8.75. Reviewed by T. D. Seeley in *Nature* **356**, 626 (1992).

Simplicity and Complexity in Games of the Intellect by Lawrence B. Slobodkin. Harvard, \$12.95, £10.25.

Rockets into Space by Frank H. Winter. An overview of the history of aeronautics. Harvard, \$12.95, £10.25.

Planet Earth: A View from Space by D. James Baker. An introduction to remote sensing. Harvard, \$12.95, £10.25.

The Maya by Michael D. Coe (5th edn). A clear and readable introduction. Thames and Hudson, £7.95.

The Third Revolution: Population, Environment and a Sustainable World by Paul Harrison. Penguin, £6.99, \$12.

Famous sleepers

Jim Horne

Why We Nap: Evolution, Chronobiology, and Functions of Polyphasic and Ultrashort Sleep. Edited by Claudio Stampi. Birkhäuser: 1992. Pp. 280. DM188, SFR168, £71, \$99.

PERPLEXINGLY, and by the editor's own admission, little of this book is devoted to "why we nap" (a title that has a familiar ring to it — in 1988, Oxford University Press published my own book called *Why We Sleep*). It seems that the contributors have tended to write about what they really wanted to, rather than to follow the editor's brief. Things become clearer when one realizes that Claudio Stampi's inspiration for the book is the peculiar sleep habit attributed to Leonardo da Vinci. Unsupported legend has it that the great man eschewed much of sleep by routinely sleeping for 15 minutes every four hours, taking only some 90 minutes of sleep in 24 hours. This 'polyphasic ultrashort sleep' (as it is now called) is distinct from just having one or two naps in the daytime and a substantial sleep at night: it is the loss of the latter with many more of the former. We are reminded by Stampi that this regime has been used by other famous men such as Napoleon, Salvador Dali and Thomas Edison, all of whom, I must add, were not typical of us mortals and, to say the least, were somewhat eccentric and perhaps tended towards hypomania, a disorder that has short sleep as a symptom. So we might be gratified that 'famous women' are absent from Stampi's list.

Stampi is also a keen yachtsman, and advocates polyphasic ultrashort sleep for long-haul racing. Unfortunately, not many of the other contributors share his enthusiasm for this form of sleep, and

concentrate on the more common findings associated with minimal sleep requirements and the effect of the 24-hour circadian rhythm on sleep. The book is therefore rather diverse, with the editor's own commendable and provocative chapters on polyphasic ultrashort sleep and its ramifications going one way, and most of the other contributions, by well-known sleep researchers, on more familiar, broader accounts of sleep, going another. I am left with the impression that the editor could and should have written much more of the book himself.

To be more positive, one of the volume's best developed themes concerns the question "how much sleep do we need?", and in this respect the chapters by N. Ball and by P. Naitoh are particularly perceptive. Ball, an ethologist, presents a comparative perspective on the variety of sleep schedules adopted by mammals and birds to suit their niches and needs; indeed, his epistemological slant also addresses why we nap. Naitoh, a respected sage of sleep research, has in effect produced an informed and critical but constructive overview of the whole book. He is circumspect about advocating many short sleeps per day, and in doing so tends to temper the editor's enthusiasm. Naitoh points out that although Leonardo's method may be worthwhile, it will be achieved only after much effort and anguish. Initially, at least, it is not conducive to efficient sleep, as for each sleep episode one must take time to fall asleep and then to enter a worthwhile sleep. Waking up will be difficult, and a period of grogginess ("post sleep inertia") ensues. Not all of Leonardo's inventions worked, and perhaps, for polyphasic ultrashort sleep, it is back to the drawing board. □

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Our Universe in the balance

M. Disney

Principles of Physical Cosmology. By P. J. E. Peebles. Princeton University Press: 1993. Pp. 718. \$59.50, £55 (hbk); \$29.95, £19.95 (pbk).

ONLY beasts could remain indifferent to the origin, structure and fate of the cosmos in which they live. Only saints could resign themselves to never knowing the answers. The upshot has been that every civilization, almost every generation, has put together such meagre observations as it possesses, has interpreted them in the light of currently fashionable theories, and has called the subject cosmology. There can be no doubt as to the significance of cosmology. There can be grave doubts as to its status as a science. Rutherford is alleged to have growled: "Don't let me hear the word 'Universe' in this department".

The difficulties of turning cosmology into a science are manifold. There's only one Universe; all comparisons and statistical tests are rendered useless. For all but the past few of its 60 decades of temporal existence, the Universe has been opaque to electromagnetic radiation. The time frame of human science is so short, in cosmic terms, that we have in effect only a single still shot of a dynamical Universe. Worst of all, we have only about eight-and-a-half observations that have any bearing on the subject at all.

But cosmology is altogether too fascinating to be ignored. The real challenge to science is to put cosmology in the right place, which is neither in the dustbin nor on the altar. If we don't find a reasonable balance then we could waste enormous effort on futile pseudoscience, or halt progress altogether. Over the past few decades the subject has been inching forward, sometimes by design, more often by accident. In particular, the opening up of the electromagnetic spectrum is bringing, or promising to bring, interesting new observational data. But progress in no way matches the hullabaloo, often orchestrated by self-interested scientists, that gets into the media, for instance the ante-scientific way the recent results from the Cosmic Background Explorer were released at NASA's press circus.

Nothing is more badly needed than a solid but accessible book on cosmology, written by an insider who has not lost his or her scepticism. Peebles's new book fits the bill admirably. A distinguished contributor himself, one of the predictors of the cosmic background radiation, he has taken much trouble to write a scholarly book not just for the expert,

but for anyone with some undergraduate physics and a fair amount of mathematical stamina. His expertise is not in doubt, nor is his scepticism. He admits: "I have been studying cosmology for thirty years now... but it was never my plan, in fact my first reaction to cosmology was one of surprise that grown people could reasonably care about such a schematic physical theory.... I think I stuck with it [because] it is too exciting to leave". By and large though, Peebles is an enthusiast for Big Bang cosmology because he feels that "the days are gone when it is easy to think of viable alternatives".

In writing this book, Peebles has set himself three guidelines: to concentrate on results that seem likely still to be of interest in a decade; to keep out of cosmo-particle physics; and to play down computer modelling. And perhaps the last two follow from the first. In my judgement he's got it about right. This is a book I shall treasure for myself, urge upon my colleagues and graduate students, and hope that my undergraduates can afford to buy. Certainly every university library should have at least one copy, because many with a taste for cosmic physics will want to dip into it, read it or use it for reference.

There are three certain truths about cosmology. The subject is fascinating, it generates almost as much nonsense as religion and it will never be far from the headlines. For all three reasons you might like to have your copy of Peebles close at hand. □

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■ Two other textbooks in the same area, both from Cambridge University Press, were unlucky to come out at the same time as Peebles's volume — they suffer badly by comparison. Roger J. Taylor's *Galaxies: Structure and Evolution* (pp 208; £30, \$49.95 (hbk); £12.95, \$22.95 (pbk)) is an updated reissue of a book aimed at keen final-year school students. It fills a neglected niche between the popular text without mathematics and full-blooded textbooks. Large public libraries and wealthy schools should think of buying a copy. T. Padmanabhan's *Structure Formation in the Universe* (pp 483; £50, \$89.95 (hbk); £19.95, \$34.95 (pbk)) is aimed at graduate students and above, and deals with the formation of clusters and galaxies. Highly theoretical. **M.D.**

■ Cambridge University Press has also just published a revised second edition of J. V. Narlikar's *Introduction to Cosmology* (£50, \$79.95 (hbk); £17.95, \$29.95 (pbk)). For a review of the first edition see *Nature* 308, 141 (1984).

The descent of *Equus*

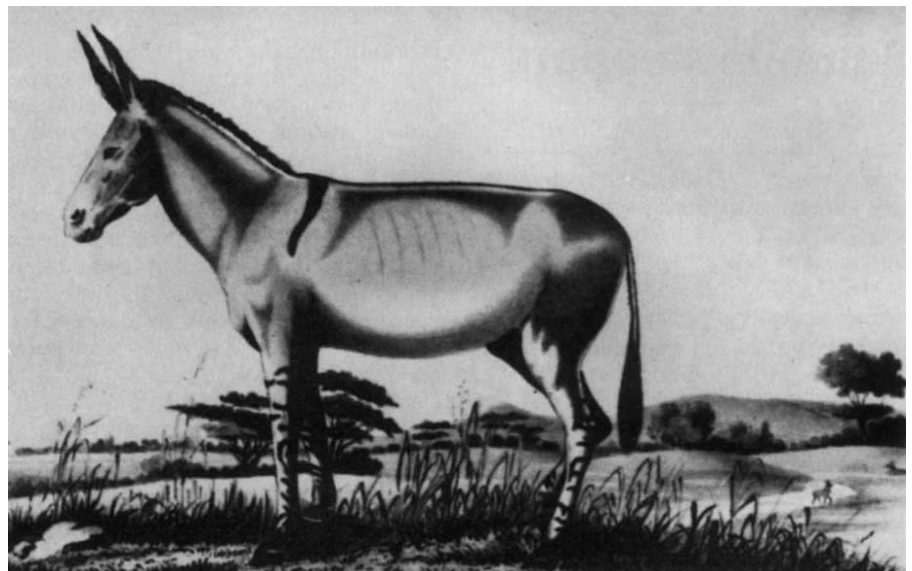
Adrian Lister

Fossil Horses: Systematics, Paleobiology and Evolution of the Family Equidae. By Bruce J. MacFadden. Cambridge University Press: 1993. Pp. 369. £45, \$74.95.

WHEN Thomas Henry Huxley, "Darwin's bulldog", visited Yale in 1876, he was enthralled by O. C. Marsh's sequence of fossil horses, from little *Eohippus* (correctly *Hyracotherium*) to modern *Equus*, forming the first clear demonstration in the fossil record of the descent of a living

occasional dwarfing, the predominant trends throughout the clade, by a combination of common descent and parallel evolution, are in the classic direction: size increase, toe reduction, elongation of tooth crowns and so on. These trends explain the success of the family as a whole, although, as MacFadden notes, they were driven by drying and the spread of grassland through the Tertiary, rather than by 'progress' through ever-better adaptation to a constant niche.

The book is arranged thematically, giving balanced coverage of taxonomy, phylogeny (a valuable summary of synapomorphies at each node of the tree), biogeography, functional morphology and palaeoecology. The history of evolutionary study receives considerable treatment; this is interesting and not



The African wild ass *Equus africanus taeniopus*, first described by Heuglin in 1861.

species. Horses have remained the most famous of fossil lineages, and, as amply demonstrated in this well-crafted book, continue to provide a testing-ground for the latest concepts in palaeontology.

As early as 1930, Matthew realized that equid evolution fitted not a single lineage but a complex branching tree, and current taxonomy recognizes about 150 fossil and living species over a 58-million-year history. Nonetheless, the portrayal of a single line leading to *Equus* persists in museum displays, textbooks and undergraduate essays. MacFadden rightly criticizes this view as misrepresenting the complexity of evolutionary history and process. On the other hand, it seems perfectly valid to ask "what is the line of direct descent leading to *Equus*?" (or any other species), provided the limitations of the question are understood. And it is interesting how often in this book, whether tracing changes in anatomy or in habitat, the discussion proceeds along the traditional line from *Hyracotherium* to *Equus*. For despite the diversification and

inappropriate given the important role of fossil horses in evolutionary and palaeontological thought. A lot of space is also taken up explaining general principles, and sometimes there is a slight imbalance; for example, 14 pages of general biogeography introduce 8 pages of horse biogeography. These passages will help students and general readers; and if specialists find that the horse meat is sometimes a little lean, there is an excellent and thorough bibliography.

Recent years have seen great advances in the study of fossil horses, particularly in phylogenetically distinguishing clades from grades, and in interpreting adaptation and ecology on the basis of analogy with feeding morphotypes among modern mammalian guilds. MacFadden has produced a timely and readable text, a good advertisement for the biological fruits that the palaeontological tree can bear. □

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Atmospheric carbon dioxide and the ocean

U. Siegenthaler & J. L. Sarmiento

The ocean is a significant sink for anthropogenic carbon dioxide, taking up about a third of the emissions arising from fossil-fuel use and tropical deforestation. Increases in the atmospheric carbon dioxide concentration account for most of the remaining emissions, but there still appears to be a 'missing sink' which may be located in the terrestrial biosphere.

HUMAN activities have led to considerable anthropogenic emissions of greenhouse gases. In particular, for the period from 1980 to 1989 carbon dioxide emissions from fossil-fuel burning¹ and tropical deforestation^{2,3} amounted to $\sim 7.0 \pm 1.1 \text{ Gt C yr}^{-1}$ (gigatonnes carbon; $1 \text{ Gt} = 10^{15} \text{ g}$). Increases in the atmospheric CO_2 concentration can account for about half of the CO_2 emissions for this period^{4,5}. The ocean has also taken up large amounts of anthropogenic CO_2 , but there have been conflicting estimates of this uptake. Models of the global carbon cycle (which calculate oceanic CO_2 uptake by simulating downward transport of CO_2 -laden water from the ocean surface) yield a mean 1980–89 oceanic CO_2 uptake of $2.0 \pm 0.6 \text{ Gt C yr}^{-1}$ (see Table 1). By contrast, Tans *et al.*⁶ estimated an oceanic uptake for the same period of $<1 \text{ Gt C yr}^{-1}$, from surface-water $p\text{CO}_2$ measurements for the Northern Hemisphere and the interhemispheric difference in atmospheric CO_2 concentrations. This led Tans *et al.* to propose the existence of a large 'missing sink' for CO_2 , probably located in the terrestrial biosphere. It is clearly necessary to settle the question of how much CO_2 is absorbed by the oceans, before any conclusions can be drawn about the

presence and importance of other natural sinks.

Here we evaluate the various estimates of the amount of anthropogenic CO_2 taken up by the ocean that have been published in recent years. We show that the estimate of Tans *et al.* neglects several important processes, and conclude that available evidence to date supports an annual mean uptake of $\sim 2 \text{ Gt C yr}^{-1}$. Having established a 'best guess' for the oceanic CO_2 uptake, one can then show that the total accumulation of CO_2 in the atmosphere and ocean from 1980 to 1989 is significantly less than current estimates of the total CO_2 emitted during that period. This implies either that the CO_2 release from tropical deforestation is much smaller than currently estimated, or that there is indeed a 'missing sink' for the CO_2 , albeit smaller than the one proposed by Tans *et al.* Future studies are needed to improve our understanding of the magnitude and location of these respective CO_2 sinks, if we are to predict future changes in atmospheric CO_2 .

Global carbon cycle and the atmospheric record

Figure 1 shows the global carbon reservoirs and fluxes for pre-

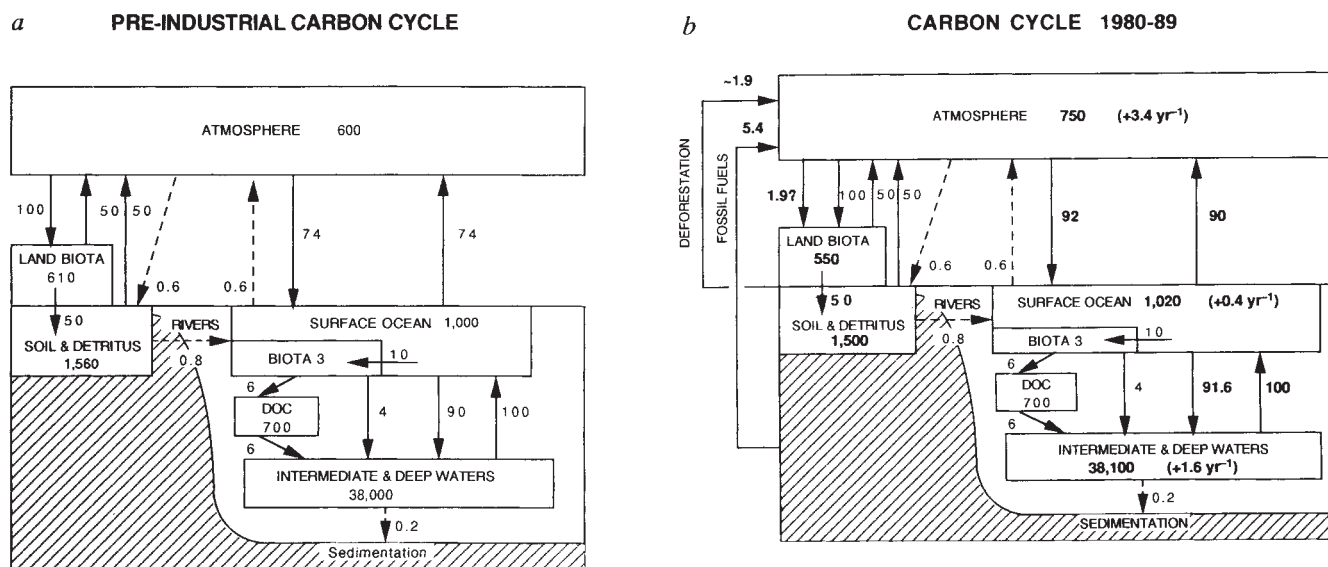


FIG. 1 Global carbon cycle reservoirs and fluxes, in Gt C and Gt C yr^{-1} , respectively ($1 \text{ Gt C} = 10^{15} \text{ g C}$). *a*, Reconstructed pre-industrial situation and *b*, present-day situation. In *b*, bold numbers denote fluxes or reservoir sizes which have changed due to human activities. The numbers in *b* correspond approximately to those given in the 1990 IPCC assessment² with the following exceptions: an oceanic pool of dissolved organic carbon (DOC) is included (E. Peltzer, personal communication). The marine biological new production (equal to particles plus DOC exported from the surface) is 10 Gt C yr^{-1} , taken from model calculations^{65,66}. The indicated transport by water circulation is much larger than in the IPCC assessment², but this is primarily a matter of

definition. The IPCC downward flux of 35 Gt C yr^{-1} corresponds roughly to global deep-water formation ($\sim 46 \times 10^6 \text{ m}^3 \text{ s}^{-1}$). Our upward flux (100 Gt C yr^{-1}) is chosen such that it is about ten times the total new production, which in a 2-box model yields a surface water ΣCO_2 deficit of 10%, as observed. Our fluxes therefore represent exchange between the surface and a depth of perhaps 1 km where most of the particles and DOC have been remineralized. The cumulative land-use effect, assumed to be -120 Gt C , is divided equally between vegetation and soils. The difference between river input and sedimentation has been closed by fluxes of 0.6 Gt C yr^{-1} from ocean to atmosphere and from atmosphere to biota (dashed arrows).

TABLE 1 Estimates of the average oceanic CO₂ uptake rate (Gt C yr⁻¹)

Model number and type*	Oceanic uptake rate†
1. Box-diffusion model (refs 55, 82)§,‡	2.32
2. HILDA (ref. 35)§	2.15
3. Princeton Ocean General Circulation model (ref. 28)¶	≥1.9
4. Hamburg Ocean General Circulation model (M. Heimann & E. Maier-Reimer, personal communication)§	1.58

These estimates are for 1980–89, based on different carbon cycle models, normalized to a common mean penetration depth z for bomb-¹⁴C (z = (column inventory/surface concentration), refs 80, 81, calculated using the bomb-¹⁴C inventory or ref. 32).

* Models 1 and 2 are simple, parameterized ocean models. 1 is purely one-dimensional; 2 is 1.5-dimensional with a subdivision into low- and high-latitude ocean (HILDA = high-latitude exchange/interior diffusion-advection model); 3 and 4 are three-dimensional models.

† Oceanic uptake rate obtained by deconvolving the CO₂ concentration record (Siple ice core data^{15,83} and Mauna Loa observations¹³, that is by prescribing the atmospheric concentration as a function of time and calculating the corresponding flux into the ocean.

‡ Goudriaan^{84,85} used a similar model to the box-diffusion model, but with two separate ocean basins. His model was not run in the deconvolution mode, but forced by prescribed anthropogenic emissions, and the simulation did not include the period 1980–1989; Orr³³ corrected for this and obtained 2.2 Gt C yr⁻¹. However, the information about the bomb-¹⁴C simulation used is Goudriaan's model is insufficient to perform a normalization.

§ The mean bomb-¹⁴C penetration depth is 343 m from observations³² (derived from GEOSCECS data, nominally 1974). The simulated penetration depth is correct for the HILDA model, version K(2); 5% too large for the box-diffusion model; 8% too small for the Hamburg model. The listed uptake values are correspondingly corrected.

¶ The Princeton model has too low a bomb-¹⁴C penetration depth³¹, so that the oceanic CO₂ uptake in this model is considered as a lower limit (see ref. 28 for details).

industrial (Fig. 1a) and present (Fig. 1b) conditions. The largest pool is dissolved inorganic carbon (ΣCO₂) in the ocean, including CO₂, HCO₃⁻ and CO₃²⁻. Carbon dioxide is exchanged between atmospheric and ocean by gas transfer, and between the biota and the other reservoirs by photosynthesis and respiration. In pre-industrial times, the carbon cycle was in a roughly steady state, with the sum of all fluxes into each reservoir being equal to the sum of the fluxes out. Because of anthropogenic carbon emissions, fluxes and carbon reservoir sizes changed (bold-face numbers in Fig. 1b). The atmospheric concentration of CO₂ has increased from its pre-industrial value of 280 parts per million (p.p.m.) to 355 p.p.m. in 1992. The cycle we observe today represents a superposition of the natural situation and man-made perturbations. The recoverable fossil-fuel resources are estimated at perhaps 4,000 Gt C (ref. 7), a huge amount compared to the atmospheric reservoir (600 Gt C) and to the live vegetation (~550 Gt C). The continued combustion of fossil fuels has the potential to raise atmospheric CO₂ levels several fold, whereas the potential effect of continued deforestation is limited.

The atmospheric CO₂ concentration has been measured continuously at Mauna Loa and at the South Pole since 1958 (Fig. 2a, refs 4, 5), and later at many other monitoring stations⁶. For earlier times, the atmospheric CO₂ concentration can be reconstructed by means of analysis of air trapped in old polar ice (Fig. 2b). This concentration has grown steadily since AD 1800, which has given rise to a net CO₂ uptake by the oceans and probably by parts of the land biota. The atmospheric growth is due to emissions from combustion of fossil fuels, 5.4 Gt C yr⁻¹ for the decade 1980–1989 (refs 1, 9), and from deforestation and land use, estimated at 0.6–2.5 Gt C yr⁻¹ (refs 2, 3). The large range of the estimates of deforestation flux^{10,12} reflects the fact that

TABLE 2 Budget of annual anthropogenic CO₂ perturbations

Emissions from fossil combustion ¹	5.4 ± 0.5
Emissions from deforestation and land use ^{2,3}	0.6 to 2.5
Atmospheric accumulation ^{4,5}	3.2* ± 0.2
Uptake by the ocean	2.0 ± 0.6
Net imbalance	1.8 ± 1.3

These values are averages for the decade 1980–89. Emissions from deforestation and land use also include forest regrowth on abandoned land, but not a possible CO₂ fertilization. For the deforestation flux, a range instead of an error is given, because estimates from different authors cannot just be averaged to get a best value. For estimating the net imbalance and its error (obtained by quadratic addition), however, 1.6 ± 1.0 Gt C yr⁻¹ is adopted for deforestation.

* Average of Mauna Loa and South Pole.

terrestrial vegetation and soils are extremely heterogeneous and that it is difficult to assess changes in carbon storage per unit area cleared and regrowth of abandoned land.

The natural exchange fluxes between atmosphere, ocean and land biota are relatively large compared to the anthropogenic perturbations (see Fig. 1b). Therefore, the question has sometimes been raised whether the current increase could just be due to a natural excursion of the carbon cycle which would eventually reverse. Several arguments disprove this idea. In Fig. 2c, the annual growth of atmospheric CO₂ is compared with the estimated emissions from fossil-fuel combustion and land use. The atmospheric growth has been smaller than the fossil-fuel input in every single year from about 1900 onwards, and it has been smaller by a factor of 2–3 than the total estimated annual input since the beginning of the deforestation record. The increase from 1860 to 1989 corresponds to an airborne fraction of 40% of the estimated cumulative anthropogenic release (see legend to Fig. 2c). Thus, during all these years, ocean plus land biota together have acted as a net CO₂ sink rather than a source. A compelling piece of evidence for the relation between emissions and atmospheric increase comes from the observation that the annual mean concentration in the Northern Hemisphere, where 95% of the fossil-fuel emissions occur, has exceeded the concentrations in the Southern Hemisphere since the start of atmospheric monitoring (Fig. 2a) and that the interhemispheric difference has grown in parallel to the fossil-fuel emissions^{13,14} (Fig. 2d).

Is it possible that natural fluctuations have modulated the concentration change? During most of the Holocene period and also during the last interglacial, the atmospheric level of CO₂ was near 280 p.p.m. (refs 15–17). Variations in CO₂ levels comparable in magnitude to the anthropogenic increase are documented only for periods with drastic climatic changes, such as the transition from the last glacial to the Holocene^{15,17}. Analyses of ice cores from the South Pole, covering the time AD 1000–1800 (Fig. 2b) (ref. 18), and from other sites^{19,21} indicate that between AD 1000 and 1800, the CO₂ concentration varied within about 10 p.p.m. This variability is much less than the increase since AD 1800. It may not seem obvious that the natural fluxes are so well balanced when considering that the atmosphere exchanges more than 10% of its carbon mass every year with both the land biota and the ocean. But a large fraction of the gross fluxes consists of molecules circulated rapidly between relatively small sub-reservoirs.

On a shorter time scale, interannual variations of about 1 p.p.m., corresponding to a transfer of ≤2 Gt C yr⁻¹ into or out of the atmosphere, have been observed, correlated with El Niño Southern Oscillation (ENSO) events^{13,22,23}. They seem to be connected to carbon releases by land vegetation, due possibly to ENSO-related drought and the increased frequency of fires^{13,24}. There is good evidence that the natural atmospheric CO₂ variations are relatively small under present climatic condi-

tions. Thus, the assumption that the natural cycle has operated in a steady state in the past and continues to do so, upon which the models of the global carbon cycle discussed below are all based, seems to be a reasonable approximation.

Uptake of CO₂ by the ocean

The ocean is the largest of the rapidly exchanging global carbon reservoirs (Fig. 1) and a major sink for anthropogenic carbon. The increase of dissolved inorganic carbon (ΣCO_2) in the oceans is difficult to measure experimentally, because the annual increase is small compared to the regional and temporal variability. Therefore, the oceanic CO₂ uptake has to be estimated using ocean-atmosphere carbon-cycle models. The net air-sea flux is driven by the difference in partial pressures of CO₂ and can be written as

$$F = k_g(p\text{CO}_{2,a} - p\text{CO}_{2,s}) = k_g\Delta p\text{CO}_2 \quad (1)$$

where $p\text{CO}_{2,s}$ is the equilibrium partial pressure of CO₂ in surface sea water, $p\text{CO}_{2,a}$ is the partial pressure of CO₂ in the air and k_g is a gas exchange coefficient. Whereas the air-sea exchange occurs by means of gaseous CO₂, most of the ΣCO_2 in surface sea water is in ionic form and less than 1% is dissolved CO₂ gas. When sea water takes up additional CO₂, ΣCO_2 and $p\text{CO}_2$ both

increase, but the chemical equilibria between the carbon species (CO₂, HCO₃⁻ and CO₃²⁻) are shifted such that the relative increase of $p\text{CO}_2$ is about 10 times larger than that of ΣCO_2 . This buffer mechanism determines the uptake capacity of the ocean: the ocean contains about 65 times more carbon than the pre-industrial atmosphere, but at equilibrium it can take up only ~6.5 times as much additional carbon as the atmosphere. This capacity becomes available only when the whole ocean is chemically equilibrated with the new atmospheric CO₂ concentration, which would take roughly 1,000 yr, the turnover time of the deep sea. The dynamics of the oceanic uptake of CO₂ is therefore strongly determined by the rate of downward transport of CO₂-laden water from surface to depth. The gas-exchange rate is fast enough to ensure that surface-water $p\text{CO}_2$ is near equilibrium with the atmosphere (except in ocean regions where vertical water exchange is vigorous), with a global mean $\Delta p\text{CO}_2$ of currently ~8 p.p.m., which is small compared to the atmospheric increase of ~75 p.p.m. since pre-industrial times. The global mean $\Delta p\text{CO}_2$ is so small because surface sea water reaches chemical equilibrium with the atmospheric CO₂ concentration within typically one year²⁵, whereas the timescale for downward transport from the surface to deeper layers in the ocean is considerably longer. Thus, the main rate-determining step for oceanic

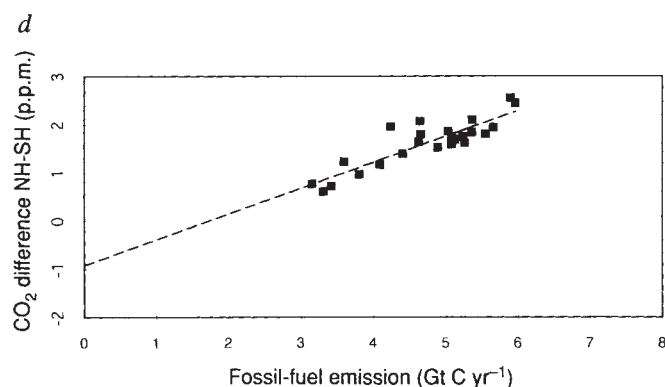
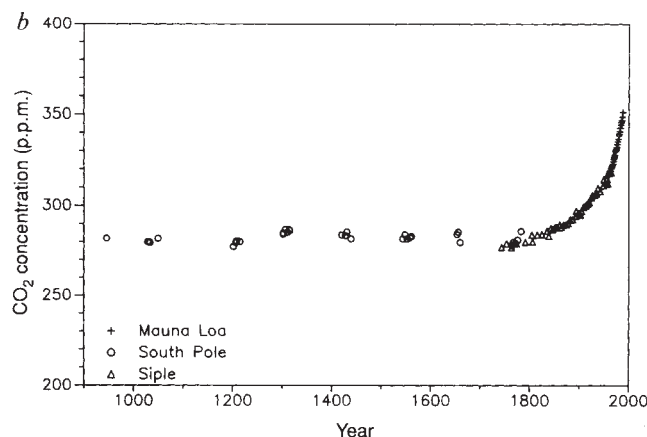
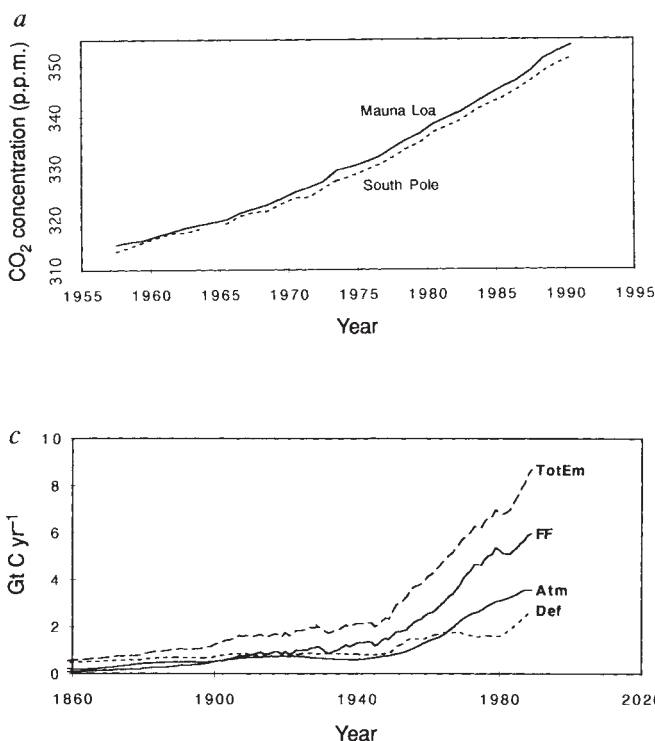


FIG. 2 a, Atmospheric CO₂ measurement at Mauna Loa, Hawaii⁵ and at the South Pole⁴. b, Record of atmospheric CO₂ for the past 1,000 yr, given by direct atmospheric observations at Mauna Loa⁵ (+), measurement on Antarctic ice cores from Siple station^{15,83} (Δ) and South Pole¹⁸ (O). c, Annual growth of amount of CO₂ in atmosphere (Atm), emissions from fossil fuel use^{1,9} (FF), an estimate of net flux due to deforestation and land use¹¹ (Def) and the sum of both emissions (TotEm). The year-to-year increase in the atmosphere has been smaller than the annual emissions from fossil fuels since about 1900 and than the total estimated annual emissions since 1850, that is the start of the deforestation record. The annual atmospheric CO₂ increase observed in the Siple ice core before 1900 cannot be explained by the fossil emissions alone, which indicates that carbon release from deforestation was significant in the 19th century, as also obtained from the direct estimates. For the whole period 1860 to 1989, the total estimated CO₂ release from fossil fuels and deforestation is about 345 Gt C, while the atmospheric increase amounted to 138 Gt C, corresponding to an average airborne

fraction of 40% of the cumulative release. (The ratio of atmospheric increase to cumulative fossil input only is 65%; our figure includes the contribution from deforestation.) The airborne fraction has varied in time, depending on the history of the emissions. For the decade 1980–1989, it is $46 \pm 8\%$. d, Interhemispheric CO₂ concentration difference (Mauna Loa–South Pole^{4,5}) versus global fossil-fuel derived emissions. The Northern Hemisphere–Southern Hemisphere difference is the result of a balance between emissions, interhemispheric transportation through the atmosphere and surface sinks. The good correlation can be explained by the fact that most of these emissions (95%) take place in the NH. Note that if extrapolated to zero fossil-fuel emissions, the SH concentration would be ~1 p.p.m. greater than that in the NH, which, comparing with the model calculations of refs 6, 13 roughly correspond to a northward flux of ~1 Gt C yr⁻¹.

CO₂ uptake, which must be correctly simulated by carbon-cycle models, is the vertical water transport. The time needed for penetration of anthropogenic carbon into the thermocline (topmost 1,000 m) is several decades, and many centuries are needed to reach the large reservoirs of the deep sea. The behaviour of the oceanic uptake over time can be studied in a model by the progressive decay of a hypothetical CO₂ pulse into the atmosphere^{26,28}. The model-calculated CO₂ uptake, to the present, corresponds typically to an average penetration depth of 600 m.

The models used vary in complexity from simple box-type schemes (for example refs 29, 30) to three-dimensional Ocean General Circulation Models (for example refs 27, 28). In the former, the calculation of oceanic circulation is not based on physical laws, but is prescribed in a parametric fashion, with the oceanic transport parameters determined by the use of observations of a suitable tracer such as bomb-produced ¹⁴C. Verification of the simulated pattern and rate of circulation by means of suitable tracers is essential also for three-dimensional models³¹. The results for the oceanic uptake, obtained by different models from a deconvolution of the Siple-Mauna Loa CO₂ record and normalized by the penetration depth of bomb-¹⁴C, are all in the range 1.6–2.3 Gt C yr⁻¹ (Table 1). Assuming an error ±20% for the bomb-¹⁴C inventory³² and further that the CO₂ uptake scales directly with the bomb-¹⁴C uptake (both conservative assumptions), we estimate an overall error of ±30%. We conclude that the best model-based estimate for the current oceanic uptake is 2.0 ± 0.6 Gt C yr⁻¹ (subjectively estimated confidence 90%).

Calculating air-sea CO₂ fluxes from observations. Local, instantaneous air-sea CO₂ fluxes can be estimated from equation (1), using measured air-sea *p*CO₂ differences and estimated gas exchange coefficients. Some oceanic regions such as the Equatorial Pacific release large amounts of CO₂ to the atmosphere, other regions take up CO₂ because of undersaturation. It is important to recognize that the observed distribution of source and sink regions is given by the superposition of natural and anthropogenic processes, with the natural features generally dominating the pattern. Therefore, Δ*p*CO₂ measurements do not indicate where anthropogenic CO₂ is mainly taken up, as they only yield the total flux; the net uptake of anthropogenic CO₂ (the perturbation flux) equals the present minus the pre-industrial flux. In fact, the net sequestration is larger in the Equatorial Pacific (an outgassing region) than, for example, in the subtropics, because the water that upwells at the equator is less CO₂-contaminated than surface water in the subtropical gyre regions^{27,28}.

One problem with equation (1) is that the (wind-speed dependent) gas exchange coefficient *k_g* is not precisely known. There is a discrepancy of a factor 1.5–2 in the global mean value of *k_g* as obtained by two different methods. Estimates based on the oceanic inventories of natural or bomb-produced ¹⁴C yield an average gross exchange flux of 15–18 mol m⁻² yr⁻¹ at *p*CO₂ = 280 p.p.m. (refs 32, 34, 35), whereas a global integration of results from laboratory and field gas-exchange experiments^{36,37}, using observed winds, yields 8–11 mol m⁻² yr⁻¹ (ref. 38). The reason for this discrepancy is not clear. One suggestion is that very high exchange fluxes occur during long-lasting heavy-wind events which are not represented in the laboratory and in most field studies³⁹. Another possibility is that compared to other gases, the gas exchange rate of CO₂ is enhanced by the enzyme carbonic anhydrase which accelerates the hydration of carbon dioxide in living cells by many orders of magnitude⁴⁰. But it is not clear whether this enzyme is present in sufficient concentrations in the open ocean⁴¹.

In principle, the net oceanic uptake of CO₂ could be obtained by globally integrating local instantaneous air-sea fluxes, calculated from measured *p*CO₂ values using equation (1), $F = k_g \Delta pCO_2$. But this method is rather inaccurate. First, the variation of surface-water *p*CO₂ in space and time is much larger than the global mean Δ*p*CO₂ of (currently) ~8 p.p.m. For

instance, *p*CO₂ exhibits a temperature-driven seasonal variation of ~80 p.p.m. near Bermuda¹⁴, and biologically-driven spatial variations of several 10 p.p.m. were observed within <100 km in the northeastern Atlantic³⁷. Therefore, a spatially and seasonally very dense *p*CO₂ data coverage would be required for a global integration of equation (1). Another serious problem is that Δ*p*CO₂ is the small difference between two large numbers, so that the computed net fluxes are very sensitive to small experimental errors in *p*CO₂. Such errors may, for example, be due to uncertainties in the chemical equilibrium constants used for back-calculating *p*CO₂ from the temperature in the measuring device (equilibrator) to sea water temperature. Attempts to estimate the oceanic sink based only on Δ*p*CO₂ measurements will therefore remain rather imprecise.

While there is little hope of obtaining the oceanic CO₂ uptake from Δ*p*CO₂ observations alone, regional Δ*p*CO₂ data sets can help to constrain the atmospheric CO₂ budget (see below).

Interhemispheric concentration difference and CO₂ sinks. The discussion of the oceanic CO₂ uptake was recently stimulated by Tans, Fung and Takahashi⁶ who combined observations of atmospheric and oceanic *p*CO₂ with an atmospheric model, and concluded that the ocean takes up less CO₂ than predicted by carbon-cycle models. The atmospheric CO₂ concentration is higher in the Northern Hemisphere (NH) than in the Southern Hemisphere (SH), because 95% of the fossil-fuel CO₂ emissions occur in the NH. The observed interhemispheric difference is, however, smaller than that predicted by atmospheric transport models that employ the conventionally assumed geographical distribution of natural and anthropogenic sources and sinks (refs 6, 42–44 and P. Kasibhatla, personal communication). This indicates that the ocean in the SH takes up less CO₂ than expected, resulting in a smaller NH–SH transport and thus a smaller interhemispheric concentration difference. For a quantitative assessment, Tans *et al.* estimated the oceanic uptake in the NH by means of Δ*p*CO₂ data and equation (1); the data coverage in the SH is inadequate to do this. They then adjusted the SH oceanic uptake and introduced a NH terrestrial sink such that the observed CO₂ distribution was reproduced by the transport model. (Δ*p*CO₂ measurements are used here to determine the flux only for a restricted area of the global ocean, and the final result is therefore less sensitive to the accuracy of Δ*p*CO₂ than a global integration discussed above.) In this way Tans *et al.* obtained a total oceanic uptake of 0.3–0.8 Gt C yr⁻¹, much less than the estimate from carbon-cycle models. In order to balance the budget, they postulated a large carbon sink in the NH land biota. In contrast, Keeling *et al.*⁴⁴ have dismissed that possibility, based on atmospheric δ¹³C observations.

The result of Tans *et al.*⁶ requires revision in three respects^{45,46}. First, the skin temperature at the sea surface is (on average) a few tenths of a degree cooler than the bulk water temperature at which *p*CO₂ is determined, so that the mean surface *p*CO₂ is overestimated by several p.p.m. Robertson and Watson⁴⁷ estimated that the value of the air-sea flux north of 15° S, obtained by Tans *et al.* on the basis of Δ*p*CO₂, (and consequently their result for the global ocean) has to be increased by ~0.44 Gt C yr⁻¹ to correct for the skin effect. Second, the net air-sea gas exchange is the sum of the anthropogenic flux and a non-zero natural flux. Rivers transport organic and inorganic carbon from soils to the oceans (~0.8 Gt C yr⁻¹, see Fig. 1); a large part of this carbon was originally taken up from the atmosphere by photosynthesis on land. Part of the riverine load goes to the sediments, but a substantial fraction of the organic input is remineralized in the sea to inorganic carbon. In a steady state, the cycle must be closed by an outgassing flux of 0.4–0.7 Gt C yr⁻¹ from the ocean to the atmosphere^{45,48}. This amount has to be added to the calculated gas transfer to obtain the total anthropogenic CO₂ flux to the sea (equal to the difference between the present and pre-industrial situations). Third, there is a north-south transport of carbon in the atmosphere not only by carbon dioxide, but also by carbon monoxide. If properly accounted for, this adds

$\sim 0.25 \text{ Gt C yr}^{-1}$ to the oceanic uptake⁴³. The mean estimate of Tans *et al.* for the oceanic CO_2 uptake, revised for these three corrections, is then $0.55 + 0.44 + 0.55 + 0.25 \sim 1.8 \text{ Gt C yr}^{-1}$ (with an uncertainty difficult to estimate), in agreement with the carbon-cycle models. A recent reassessment⁴⁹ of the Tans *et al.* calculation obtained an oceanic carbon uptake of $2.0 \pm 1.0 \text{ Gt C yr}^{-1}$.

These corrections do not explain why the observed interhemispheric CO_2 difference is smaller than the value predicted by models that use the conventional distribution of sources and sinks. Interestingly, an extrapolation of the NH-SH difference to zero fossil-fuel emissions (Fig. 2d) indicates that without emissions, the SH concentration would be $\sim 1 \text{ p.p.m.}$ higher than that in the NH. This difference, compared to model calculations^{6,13}, would give rise to an interhemispheric flow of approximately 1 Gt C yr^{-1} . Model results^{44,50} suggest that this natural interhemispheric difference may partly (but probably not fully) be caused by a covariance of seasonal concentration variations and atmospheric transport patterns. Thus, an atmospheric transport of order 1 Gt C yr^{-1} across the equator seems to have existed in pre-industrial time, which must have been balanced by an equal southward flow through the ocean⁴⁴. A net southward carbon flux is plausible via North Atlantic Deep Water, carrying more carbon southward than the CO_2 -depleted surface water transports northward, although geochemical esti-

mates of this flux are smaller than expected (0.4 Gt C yr^{-1} and 0.6 Gt C yr^{-1} , refs 51, 52).

In the near future, it should become possible to improve and enhance the $p\text{CO}_2$ data base. Field programmes, for example within the Joint Global Ocean Flux Study (JGOFS), will produce large numbers of high-precision observations. The combination of atmospheric and oceanic CO_2 measurements with atmospheric models will then result in a more accurate assessment of the carbon budget, with the $\Delta p\text{CO}_2$ measurements serving as constraints on the regional air-sea fluxes. In addition, observations of transient tracers (^{14}C , freons) in the ocean obtained in the World Ocean Circulation experiment (WOCE) will provide integrated information on the rate of oceanic water fluxes; these measurements are essential for validating ocean models.

Other methods for estimating the oceanic CO_2 uptake. Carbon dioxide from the burning of fossil fuels or biomass is isotopically labelled, compared to atmospheric CO_2 , by low $^{13}\text{C}/^{12}\text{C}$ ratios. Correspondingly, $^{13}\text{C}/^{12}\text{C}$ has decreased in the various carbon reservoirs, which can be used as an additional constraint on the budget of CO_2 perturbations. Quay *et al.*⁵³ made use of this by considering the partitioning of anthropogenic $^{13}\text{CO}_2$ between atmosphere, ocean and land biota in the period 1970–1990. The atmospheric ^{13}C decrease is known from measurements. The change in the oceanic amount of ^{13}C carbon comes about by a change in isotopic composition (measured) and by the increase of ΣCO_2 . Analogously, the biotic share in the isotopic perturbation is due to a change of $^{13}\text{C}/^{12}\text{C}$ (estimated using a biota model) and the change in net biotic carbon storage. The two unknown quantities, oceanic uptake and net biomass change, can in principle be calculated if the other quantities can be determined. In this way, Quay *et al.*⁵³ estimated an average oceanic uptake for 1970–1990 of $2.1 \pm 1.5 \text{ Gt C yr}^{-1}$ (2σ error) and a net biotic carbon flux of near zero. The error stems from two main sources. First, the existing oceanic and atmospheric ^{13}C measurements are insufficient to precisely determine the small isotopic changes; further high-precision ^{13}C measurements in the atmosphere and the ocean have to be done to improve this. Second, the isotopic difference between global respiration and photosynthesis cannot be measured, but has to be estimated based on biota models. How well this can be done essentially determines the precision of the CO_2 perturbation budget achievable by this method. Although the error is presently still too large, the isotopic method represents a new and independent way to assess the oceanic CO_2 uptake.

Keeling and Shertz⁵⁴ recently published results showing the decrease of atmospheric oxygen. During photosynthesis, respiration or combustion of organic matter, about 1 mole O_2 is produced for each mole CO_2 consumed (and *vice versa*). Therefore, the atmospheric CO_2 increase is accompanied by a slight O_2 decrease. In contrast to CO_2 the ocean does not buffer the O_2 decrease, because O_2 is much less soluble than CO_2 and is not affected by aquatic chemistry. High-precision monitoring of the O_2 concentration thus allows a direct assessment of the sum of fossil-fuel and biospheric sources minus sinks of carbon. The CO_2 uptake by ocean plus land biota is then obtained by subtracting the atmospheric CO_2 increase. As the O_2 decrease is very small, $\sim 4 \text{ p.p.m. yr}^{-1}$, compared to the concentration of 210,000 p.p.m., its measurement is rather difficult. This method is only beginning; within several years, it should provide useful constraints on the carbon budget. In addition the seasonal atmospheric O_2 variations yield information on the size of the new production by the marine biota⁵⁴.

The budget of anthropogenic perturbations

Figure 3a shows the result of a model deconvolution of the Siple–Mauna Loa CO_2 record, obtained by calculating the flux into the ocean corresponding to the observed atmospheric concentration increase^{35,55}. In Fig. 3b, estimates of anthropogenic emissions are compared with the accumulation of CO_2 in the

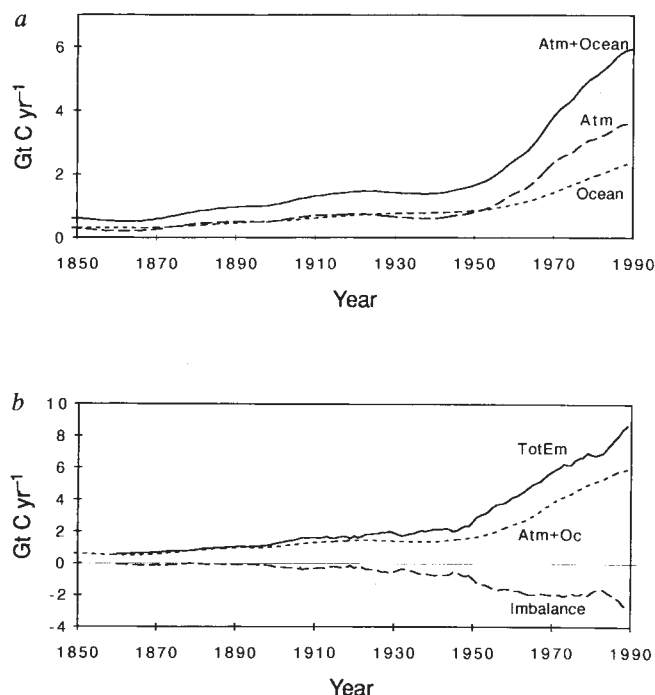


FIG. 3 a, Deconvolution of the atmospheric CO_2 record. To perform the deconvolution, the atmospheric CO_2 concentration is prescribed as a function of time (Fig. 2b) and the flux into the ocean is calculated using the HILDA model^{35,72,73}. Shown are annual CO_2 accumulations in the atmosphere (Atm; prescribed), ocean (Ocean; computed) and the sum of both (Atm + Ocean) that must be equal to the annual net flux into the atmosphere from fossil fuel combustion plus deforestation minus CO_2 fertilization of land plants plus/minus any other sources and sinks. b, Annual fluxes into the atmosphere-ocean system: estimated total anthropogenic emissions (TotEm, from Fig. 2c), CO_2 accumulation in the atmosphere plus ocean (Atm + Oc, from Fig. 3a) and difference between these quantities (Imbalance). The latter represents the imbalance or 'missing sink'; it includes the effect of stimulation of biomass growth by fertilization (by CO_2 or nitrogen emissions and so on) and from natural fluctuations. Note that the estimate for emissions from deforestation, and therefore also the imbalance, have considerable uncertainty.

atmosphere plus the ocean. The accumulation in the atmosphere plus the ocean matches the estimated emissions until about 1920, but after this there is an increasing gap between the two, indicating that either the emission estimates are significantly too high or that there is a sink not accounted for in this balance. Table 2 summarizes best estimates for the average 1980–1989 budget of the anthropogenic CO₂ perturbations. Of the estimated annual release of 7 Gt C, 3.2 Gt C yr⁻¹ remained in the atmosphere, as determined by direct measurements, and the ocean is estimated to have taken up 2.0 Gt C yr⁻¹. This leaves 1.8 ± 1.3 Gt C yr⁻¹ of the emissions unaccounted for. Although the error limits on this number are large, it seems difficult to reconcile the estimates for sources and sinks; there seems to be a 'missing sink'. The oceanic uptake is well known from the results of carbon-cycle models, so the solution to this problem must lie elsewhere.

A recent analysis of high-resolution satellite pictures indicates that deforestation has been overestimated⁵⁶, but it seems unrealistic to assume that it was virtually zero, as required for closing the perturbation budget with the sinks of Table 2. The most plausible explanation for the imbalance is that vegetation and soils are accumulating carbon, perhaps in response to the increased CO₂ concentration—in which case this sink should have grown parallel to atmospheric CO₂—or due to man-made nitrogen compounds⁵⁷. As Fig. 3b shows, the imbalance has indeed grown with time. A stimulation of plant growth in CO₂-enriched air is well-documented in short-term laboratory experiments, but it is not clear how important this effect is in nature and over longer time periods⁵⁸. Analyses of forest growth have so far not confirmed a carbon accumulation of the required size⁵⁹, but the imbalance is only a few percent of the terrestrial net primary production of 50 Gt C yr⁻¹, and the expected small increment in carbon storage density may well have escaped observation. Indirect evidence for growing biospheric activities comes from the observation that the seasonal CO₂ amplitude at many atmospheric monitoring stations has increased significantly^{14,60}.

Another reason for the mismatch between sources and sinks could be an imbalance of the natural exchange fluxes between atmosphere and ocean. But considering that there were no major CO₂ excursions in pre-industrial time, it seems unlikely that this could explain the whole imbalance.

The role of marine biology

The marine biota act as a carbon pump by producing particulate and dissolved organic matter, which is then exported to deeper layers in the ocean where it is decomposed (Fig. 1). This provides a deep source of inorganic carbon and creates a deficit of about 10% of ΣCO₂ in surface water compared to the deep sea. The size of the export fluxes is not well known, with estimates for the particulate flux ranging from 4 to 20 Gt C yr⁻¹ (refs 61, 62). Reported measurements of dissolved organic carbon (DOC)⁶³ yielding significantly higher oceanic concentrations than previously adopted have recently been withdrawn⁶⁴. Model simulations suggest a DOC export to depth of order 10 Gt C yr⁻¹ (refs 65, 66). In the unperturbed stationary state, the biogenic fluxes are compensated by an equally large upward transport of dissolved inorganic matter by water motion, corresponding to a zero net transport (except for the small fraction that goes into the sediments). The biogenic fluxes are usually not included in carbon-cycle models designed for studying the anthropogenic perturbations. This is because according to present understanding, biological productivity is controlled by nutrients (mainly phosphorus and nitrogen), light or zooplankton grazing, but not by CO₂ concentration. Therefore, the biogenic fluxes do not sequester anthropogenic carbon, but rather act as a natural background process continuing to work as in pre-industrial times (compare Fig. 1). (New laboratory studies⁶⁷ indicate that under special conditions the growth of diatoms can be affected by the availability of CO₂, but the large-scale significance of these results remains to be assessed.)

Climatic or environmental changes might modify the natural biological fluxes, which could then feed back on the atmospheric composition. Alterations of the biological carbon pump, for example related to the documented glacial-interglacial shifts of the oceanic circulation, have been discussed as possible causes of the lower ice-age CO₂ levels^{68–70}. A particularly sensitive ocean region is the Southern Ocean where the exchange between surface and deep ocean is fast, providing a large nutrient supply from depth to the surface, while marine productivity is relatively small and does not make full use of the abundant nutrients. It has been suggested that Southern Ocean productivity might be controlled by lack of the micronutrient iron⁷¹ and that fertilizing the Southern Ocean with iron could stimulate a stronger biogenic downward export of carbon and therefore counteract the man-made atmospheric CO₂ increase. Model calculations indicate that for a business-as-usual emission scenario, iron fertilization could not stop the atmospheric CO₂ growth, even under extreme assumptions about its efficiency, but only reduce the growth rate; when the iron input ceased, CO₂ would escape again from the ocean^{72–76}. Although the role of iron as a limiting nutrient is of fundamental interest for marine biology, iron fertilization is clearly not an alternative to a control of carbon emissions.

Conceivable direct effects of climate change on the biological carbon pump include a change of productivity or a more rapid remineralization of DOC and sinking organic particles due to global warming. For large environmental changes, the composition of marine ecosystems might shift. Extreme cases of the possible influence of the marine biota on atmospheric CO₂ levels are given by model simulations which indicate that for pre-industrial conditions (unperturbed concentration of CO₂: 280 p.p.m.), a fully effective biological carbon pump, reducing surface nutrient concentrations to zero everywhere, would yield an atmospheric CO₂ level of about 160 p.p.m. Extinction of all marine life would lead to a level of ~450 p.p.m. (refs 69, 77). This can be compared to a predicted ~1,000 p.p.m. in the year 2100 (ref. 78) for the highest 1992 emission scenario of the Inter-governmental Panel on Climate Change³. Changes in the marine biota could thus significantly affect atmospheric CO₂, although their potential impact is smaller than the potential anthropogenic increase. On long timescales (millenia), interactions with marine sediments have also to be considered⁷.

Indirectly, climatic change could affect CO₂ levels in different ways. The CO₂ solubility would decrease in the case of a general warming (positive feedback on atmospheric growth). Ocean circulation could change, probably reducing the downward transport of excess carbon into the interior ocean (positive feedback). Finally, oceanic circulation influences the upward transportation of carbon and nutrients and by way of nutrients, also the biological pump, as discussed in the context of glacial-interglacial variation^{68–70} (positive or negative feedback). Preliminary model calculations suggest that significant impacts on the carbon cycle would only occur for major changes in ocean circulation.

Factors controlling future CO₂ levels

The main uncertainty about the future growth of atmospheric CO₂ levels stems from the development of anthropogenic emissions, which depend on political and socio-economic factors and cannot be predicted. For instance, there is a three-fold difference in the cumulative emissions 1990–2100 between the highest (2,127 Gt C) and the lowest (742 Gt C) of the six 1992 IPCC scenarios, all based on plausible assumptions about future developments³. These 1990–2100 emissions originate largely from fossil fuels, whereas deforestation does not play an important role (30–93 Gt C) because the biomass in tropical forests is limited. For a given emission scenario, the model predictions of future atmospheric CO₂ depend on assumptions about the 'missing sink'. Calculations based on best estimates for the sources and sinks (implying an unbalanced perturbations budget) yield higher future concentrations than calculations based on a balanced budget achieved by assuming an extra

(biospheric or oceanic) sink; the difference may be considerable, especially for low-emission scenarios⁷⁹.

To improve our ability to estimate future CO₂ concentrations, intense further research is necessary. Oceanic tracer measurements, in particular of ¹⁴C and CFCs, are needed for the validation of ocean models. The resolution of ocean carbon-cycle models has to be increased. It seems unlikely, however, that revised oceanic uptake estimates will be outside the range of current model results. An observational assessment of the net carbon fluxes to the ocean and the land biota requires high-precision observations of the spatial and temporal CO₂ and isotopic distributions in the atmosphere and the surface ocean, and a variety of models for deducing fluxes. The factors controlling marine biology are not well understood. Are the ratios in which carbon and nutrients (P, N) are built into organic tissue (the Redfield ratios) indeed constant, as generally assumed? Could CO₂ be a limiting nutrient under special circumstances? The large

oceanic data sets to be produced in programmes like JGOFS and WOCE will further progress in these areas. Important uncertainties exist about the land biota (particularly concerning growth stimulation by anthropogenic CO₂ and nitrogen compounds), reaction of photosynthesis and respiration to temperature and water cycle changes and longer term modifications of ecosystem composition due to environmental change. Finally, model studies of the anthropogenic perturbation have been based on the assumption that the oceanic carbon cycle operates in a steady state. How good this assumption is remains to be determined. □

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Crystal structure of active elongation factor Tu reveals major domain rearrangements

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The crystal structure of intact elongation factor Tu (EF-Tu) from *Thermus thermophilus* has been determined and refined at an effective resolution of 1.7 Å, with incorporation of data extending to 1.45 Å. The effector region, including interaction sites for the ribosome and for transfer RNA, is well defined. Molecular mechanisms are proposed for transduction and amplification of the signal induced by GTP binding as well as for the intrinsic and effector-enhanced GTPase activity of EF-Tu. Comparison of the structure with that of EF-Tu-GDP reveals major mutual rearrangements of the three domains of the molecule.

ELONGATION factor Tu (EF-Tu)¹ is one of the most versatile characterized biological macromolecules. To fulfil its pivotal role in bacterial polypeptide synthesis, the 44K protein recognizes and specifically binds aminoacyl-tRNA, components of the ribosome, guanine nucleotides, a magnesium ion, and another protein, elongation factor Ts (EF-Ts). It has intrinsic GTPase activity², which is greatly enhanced when EF-Tu complexed with aminoacyl-tRNA and GTP binds to programmed ribosomes³. EF-Tu is the target of several antibiotics such as pulvomycin⁴, kirromycin⁴ and GE2270 A (ref. 5), all of which interfere with its function. EF-Tu also functions as one of the subunits of the replicase of bacteriophage Q β ⁶.

EF-Tu was the first protein found to be regulated by binding of GTP and GDP (ref. 7). In its active state, switched on by GTP binding, it forms a ternary complex with aminoacyl-tRNA and transports it to the A site of the messenger RNA-pro-

grammed ribosome, whereas subsequent GTP hydrolysis leads to the release of EF-Tu-GDP from the protein synthesis machinery. The rate of GTP hydrolysis has been implicated in control of the fidelity of translation⁸. In the resulting switched-off EF-Tu-GDP form, the affinity to aminoacyl-tRNA is greatly reduced and the exchange of GDP for GTP is hindered by the slow dissociation rate of GDP from the complex. The guanine nucleotide exchange factor, EF-Ts, accelerates this dissociation and, owing to the high cellular GTP concentration, EF-Tu is retransformed back into its active GTP-bound form⁹. Thus, EF-Tu cycles between the active GTP-bound state and the inactive GDP form, resembling other guanine nucleotide-binding proteins such as the heterotrimeric mammalian G proteins and the members of the *ras* protein family^{10,11}. The conformational differences between the inactive and active forms are important for the molecular mechanisms of these signal transducing GTP/

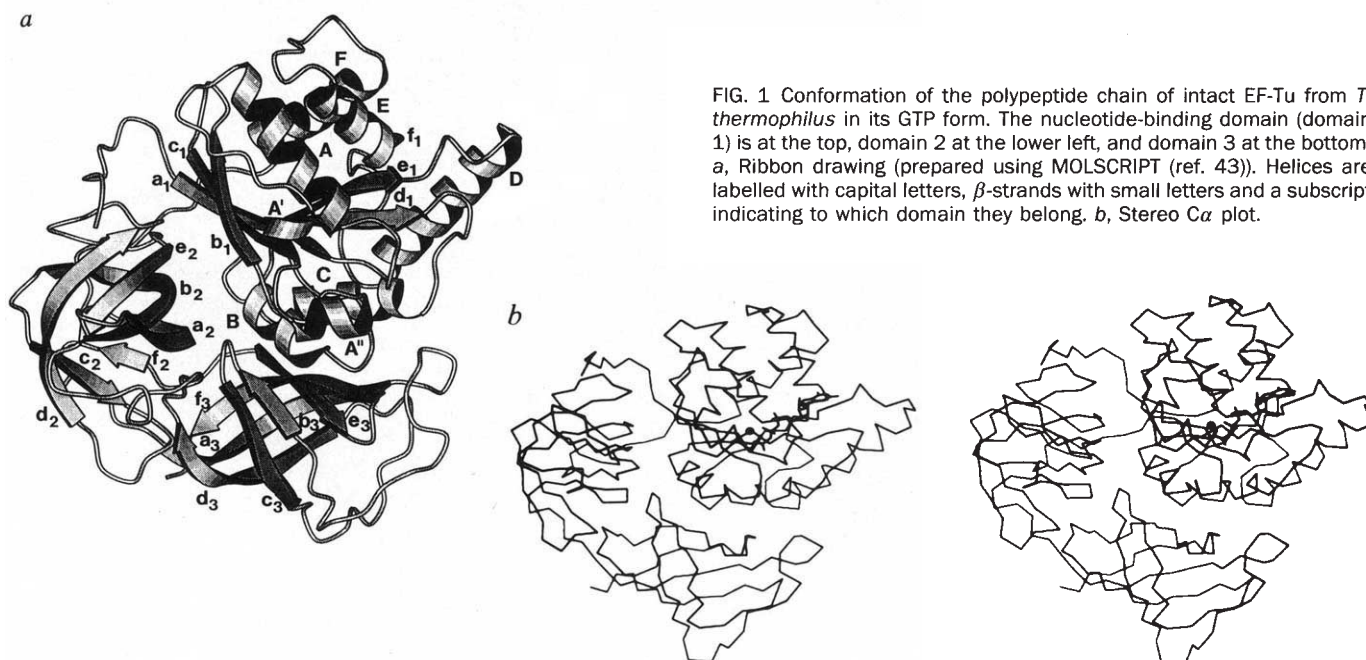


FIG. 1 Conformation of the polypeptide chain of intact EF-Tu from *T. thermophilus* in its GTP form. The nucleotide-binding domain (domain 1) is at the top, domain 2 at the lower left, and domain 3 at the bottom. *a*, Ribbon drawing (prepared using MOLSCRIPT (ref. 43)). Helices are labelled with capital letters, β -strands with small letters and a subscript indicating to which domain they belong. *b*, Stereo C α plot.

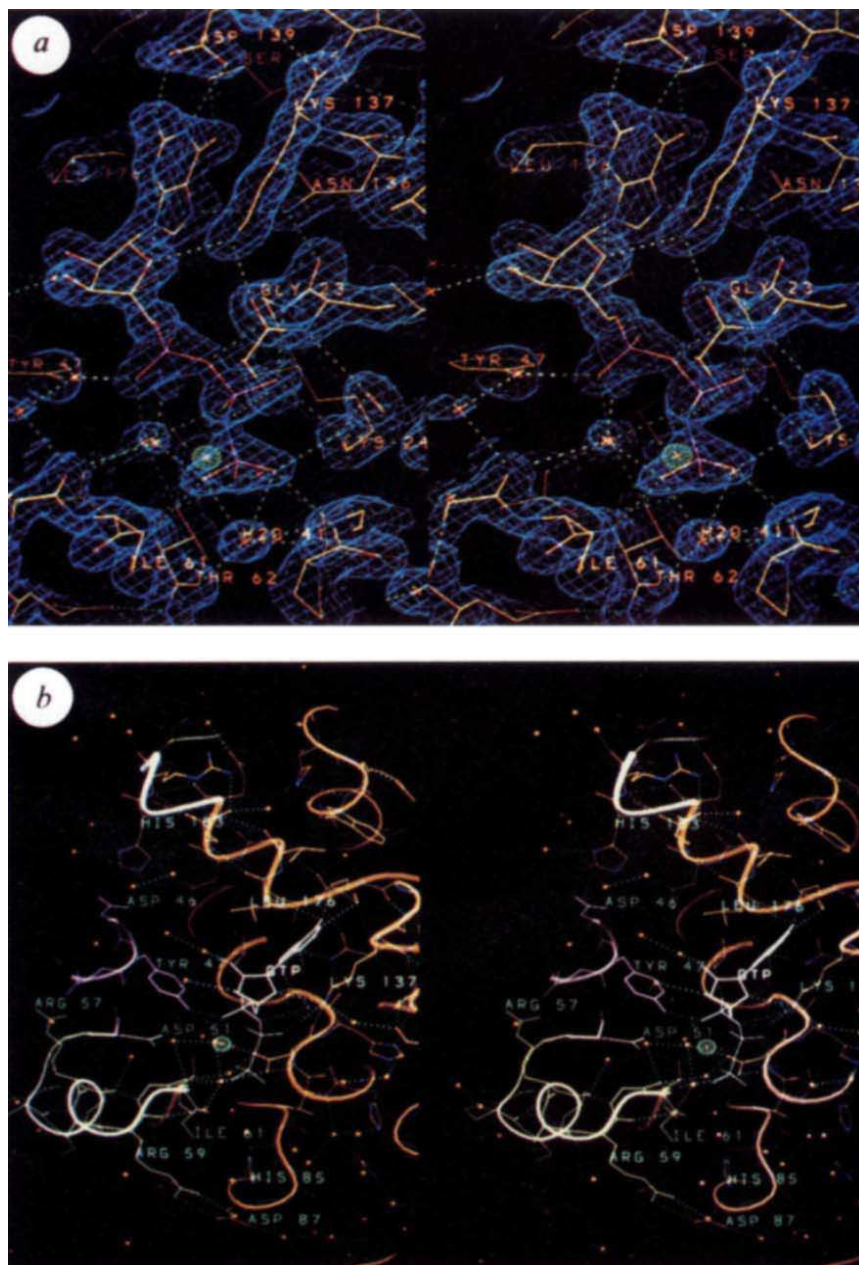
GDP-binding proteins and were studied for p21^{ras} by X-ray crystallography^{12,13}. Although p21^{ras} has been characterized structurally, its exact cellular function and identity of its downstream effector system are unclear¹⁴. In contrast, the biological function of EF-Tu is well known, allowing its structure to be correlated with the mass of available functional data. Furthermore, EF-Tu is over twice the size of p21^{ras} so it should be possible to observe conformational changes upon activation that

might propagate much further than seen in the rather small p21^{ras}.

Compared to p21^{ras}, EF-Tu is less completely defined in terms of three-dimensional structure. Crystallographic studies of the inactive, GDP-bound form of the protein at 2.9–2.6 Å resolution revealed that the molecule is organized in three domains^{15–17}. Domain 2 is separated from the nucleotide-binding domain 1 ('G domain') by an unusual hole roughly at the centre of the

FIG. 2 Stereo view of the effector region (residues 41–62) in relation to the bulk of domain 1 of EF-Tu-GppNHp. Residues in the variable portion (41–50) of the effector region (centre left) are purple, those in the constant portion (51–62; lower left) greenish-white. The position of the Mg²⁺ is indicated by a green sphere, the nucleotide is in white. a, Electron density ($2F_o - F_c$ map contoured at 1.0σ above its mean). b, Course of the main chain emphasized by a ribbon (effector region shown from residue 46 to 62). Note the exposed location of helix A' and of Arg 59 at its C terminus. A 10-residue insertion in the EF-Tu from *T. thermophilus* relative to the one from *E. coli* is depicted in white at the top of the figure. It consists partly of an extension of helix E.

METHODS. Crystals of EF-Tu-GppNHp were monoclinic, space group C2, with cell constants $a = 150.5$ (6), $b = 99.5$ (3), $c = 40.2$ (1) Å, $\beta = 95.3$ (2)°, and one complex molecule per asymmetric unit^{23,24}. Intensity data were collected initially using a FAST electronic area detector and CuK α radiation produced by a GX-21 rotating anode generator. A data set collected from 4 crystals extended to 1.9 Å resolution, with R_{sym} for individual crystals between 3.3 and 6.2% and a R_{merge} (on intensities, I) of 8.8% for reflections with $I > \sigma(I)$. Subsequently, additional data were collected from 4 crystals at wiggler beam line 9.6 of SRS Daresbury, using synchrotron radiation of wavelength $\lambda = 0.890$ Å and again a FAST area detector. These data extended to 1.45 Å and gave R_{sym} s between 4.0 and 6.7% for individual crystals. Merging all 140,538 measurements in these two data sets gave $R_{\text{merge}} = 10.0\%$ for all 58,093 unique reflections; $R_{\text{merge}} = 8.8\%$ for all reflections with $I > \sigma(I)$. The merged data set comprised 80.0% of all reflections possible to 1.7 Å resolution, and 66.1% of all reflections to 1.45 Å. Data were collected under control of the MADNES software⁴⁴ and processed using programs from the CCP4 suite⁴⁵. All data were collected at a temperature of 19 °C at the crystal. The structure was solved by a new molecular replacement approach (see Table 1). The crystallographic R factor (R_{cryst}) for the combined three-domain model oriented and translated using the parameters listed in Table 1 was 47.3% which was reduced to 44.1% after refinement at 4.0 Å resolution with the individual domains treated as rigid bodies. Further refinement used the minimization and simulated annealing features of X-PLOR^{46,47}, interrupted at intervals by inspection of 'OMIT' electron density maps⁴⁸ with coefficients $3F_o - 2F_c$ as well as of $F_o - F_c$ difference maps. Model building used the computer graphics program FRODO (ref. 49; P. R. Evans, personal communication). Resolution of data included in the refinement was gradually increased to 1.45 Å. The present model has a crystallographic R value of 20.8% for all 56,301 reflections between 8.0 and 1.45 Å, and of 19.9% for all data to 1.7 Å resolution. The r.m.s. deviations from standard bond lengths and angles are 0.015 Å and 3.1°, respectively. No residues deviate significantly from the allowed areas of the Ramachandran plot. The free R value (ref. 50) for the present model is 28.36% for 10% of the data between 5.0 and 2.0 Å resolution, well within the range expected for correct structural

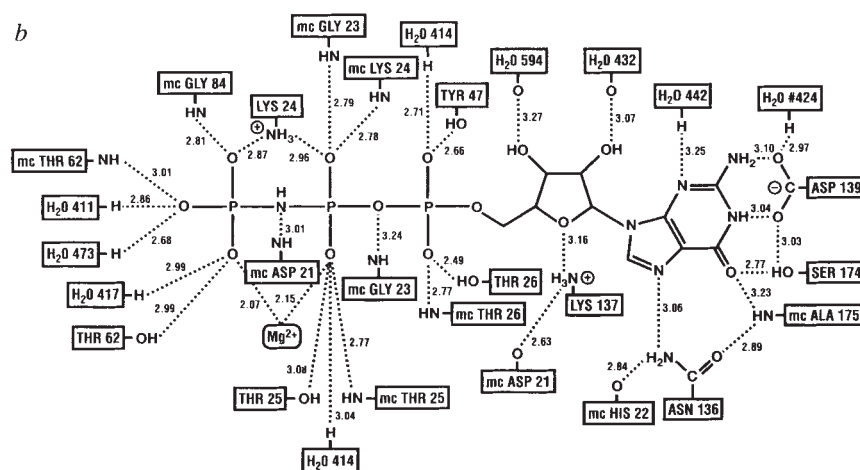
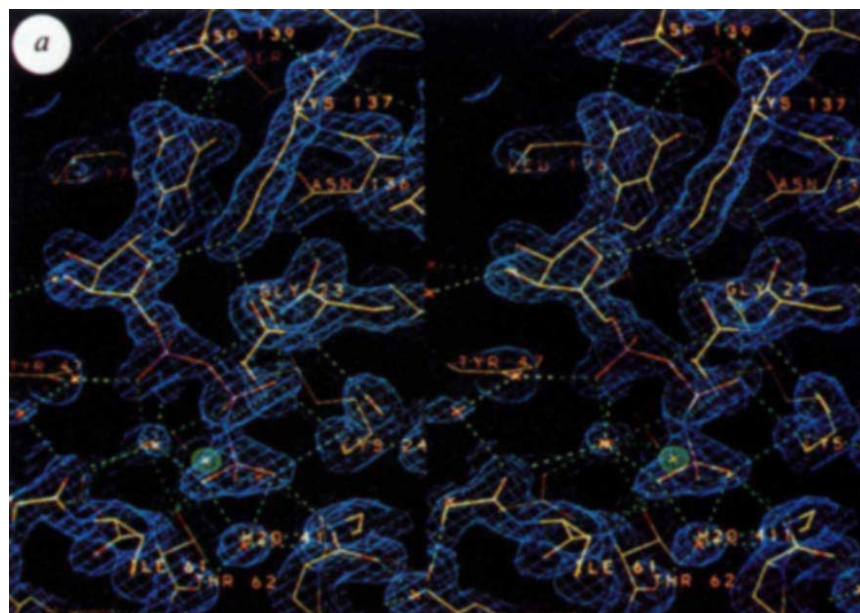


models. The final $2F_o - F_c$ map shows clear electron density for all 405 amino acids (with the exception of the distal portions of the side chains of three surface residues) and for the nucleotide as well as for the Mg²⁺ ion. In addition to the 3,236 protein and cofactor atoms, a total of 301 water molecules could be identified and included in the refined model. Coordinates will be submitted to the Brookhaven Protein Databank, where they will be held for one year. R_{sym} and R_{merge} reliability indices are defined as $R = (\sum_i |I_i - \langle I_i \rangle| / \sum_i I_i) \times 100$, where $\langle I_i \rangle$ is the average of I_i over all its symmetry equivalents. R_{sym} measures the agreement between symmetry-related reflections from the same crystal, and R_{merge} gives the overall agreement between intensities measured from different crystals. R_{cryst} , $R = (\sum_i |F_o - F_c| / \sum_i F_o) \times 100$.

molecule. Domain 1 has a tertiary structure closely resembling that of p21^{ras}. But the current model for domain 1 of EF-Tu does not comprise the full polypeptide chain. Reasonably diffracting crystals of EF-Tu from *Escherichia coli* could be grown only after mild proteolytic cleavage of the protein, which removes the oligopeptide between residues 43 and 59 (refs 15–18). The missing segment is part of the effector region of EF-Tu, that is, the site of its interaction with the ribosome¹⁹.

Because binding of aminoacyl-tRNA requires the active, GTP-bound form of EF-Tu, it was interesting to see how the nature of the bound nucleotide influences the structure of the protein. We determined the crystal structure of the EF-Tu from the thermophilic bacterium *Thermus thermophilus* in its active form, induced by complexation with the slowly hydrolysing GTP analogue, guanosine-5'-(β,γ -imido)triphosphate (GppNHp). EF-Tu from *T. thermophilus*^{20–22} shares about 70% sequence

FIG. 3 *a*, Stereo illustration of the non-hydrolysable GTP analogue GppNHp bound to EF-Tu. Green sphere, Mg²⁺ ion; red crosses, water molecules; dashed lines, hydrogen bonds; phosphorus atoms of the nucleotide are purple. Electron density is from a $2F_o - F_c$ map contoured at 1.65σ above its mean. The guanine base is recognized by hydrogen bonding with Asp 139 (top) and Ser 174 (top, in the background), and is sandwiched between Leu 176 and Lys 137. Note that there is no hydrogen bond between O6 of the base and Asn 136. 'H₂O 411' (bottom) is the active-site water molecule (see text). *b*, Schematic diagram showing the hydrogen-bonding interactions between the GppNHp nucleotide and the EF-Tu. Numbers indicate distances between non-hydrogen atoms. *c*, Environment of the active site water molecule ('H₂O 411'). Electron density is from a $2F_o - F_c$ map contoured at 1.65σ above the mean. The active-site water molecule is 'in line' with the P _{γ} -NH bond. The side chain of His 85 is separated from the active-site water molecule by the 'hydrophobic gate' formed by Val 20 and Ile 61. Dashed lines, hydrogen bonds; MG, magnesium ion.



identity with that from *E. coli*, and the two polypeptides most certainly have the same fold. We have dissected the anatomy of this form of EF-Tu at an effective resolution of 1.7 Å, using diffraction data partially extending to a Bragg spacing of 1.45 Å, and obtained a highly detailed picture of the conformational switch activated by GTP binding. Furthermore, our crystals contain the intact, uncleaved EF-Tu, so that this study defines the conformation of the effector region, segment 41–62. Finally, the X-ray structure suggests a binding site on the EF-Tu for aminoacyl-tRNA and invites speculation on a mechanism for the acceleration of the GTPase rate by interaction with its effectors.

Structure determination

Intact EF-Tu from *T. thermophilus* was crystallized as a complex with Mg^{2+} and GppNHp as described^{23,24}. The structure was solved by a new molecular replacement approach (Table 1) and refined using data extending to 1.45 Å, but because the diffraction data set is less complete beyond a Bragg spacing of 1.7 Å, the latter is our conservative estimate of the effective resolution of the model. This model has an *R* factor of 19.9% (for all data to 1.7 Å) and includes all 405 amino acids of EF-Tu, the GTP analogue (GppNHp), a Mg^{2+} ion, and 301 water molecules.

Description of the overall structure

The polypeptide chain of *T. thermophilus* EF-Tu is folded into three domains (Fig. 1). Residues 1–211 form the nucleotide-binding domain, the overall structure of which is the same as in

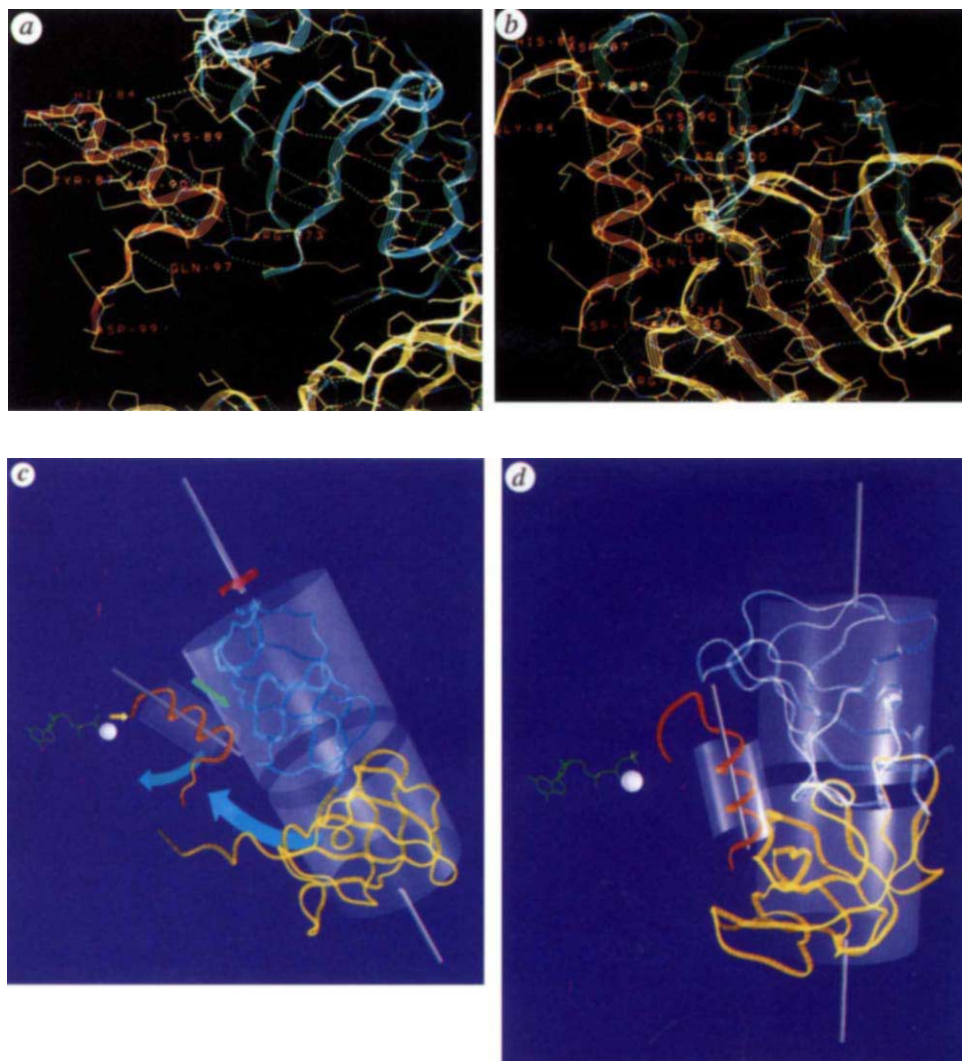
nicked EF-Tu-GDP from *E. coli*^{15, 17} and in p21^{ras} (refs 12, 25), and the same α/β topology probably exists in many other GTP/GDP-binding proteins. The central feature of this domain is a β -sheet consisting of five parallel strands (a, c–f) and one (b) running antiparallel to them. The sheet is surrounded on both sides by a total of six major α -helices (A–F), and there are 10 loop-like segments connecting the elements of secondary structure.

Apart from the overall similarity of this G domain (domain 1) to that of the trypsinized EF-Tu from *E. coli*¹⁷ there are a number of differences in detail illustrating the switch between the GTP and the GDP states of the protein. This structural switch is propagated into domains 2 and 3, which have no homologues in the *ras* proteins. Domains 2 (residues 212–311) and 3 (residues 312–405) both consist mainly of antiparallel β -strands. They pack against domain 1, and there is no hole separating the latter from domain 2, in contrast to what has invariably been seen in the GDP state, irrespective of the crystal form studied^{15,17,26}. The presence of the hole in the diphosphate form of EF-Tu does not depend on the proteolytic cleavage because it has also been observed in crystals of intact *E. coli* EF-Tu-GDP (J. Nyborg, personal communication).

The effector region (residues 41–62)

Leaving the A helix at Glu 38, the polypeptide chain makes a sharp β -turn in our structure. The chain then runs antiparallel to the A helix in an extended conformation, until it enters, at

FIG. 4 Interaction of helix B of domain 1 (orange) with domains 2 (yellow) and 3 (blue). Residues of helix B and of domains 2 and 3 are shown. Dashed lines, hydrogen bonds. Note the differences in orientation and length of the B helix in the GDP form¹⁷ (a) and in the GTP form (b) of EF-Tu, and the fact that domain 2 (yellow) is well separated from helix B in the GDP but not in the GTP complex. Figures prepared after superimposing the guanine bases of the nucleotides in the two structures. c, Schematic illustration of the portions of the GDP complex of EF-Tu (ref. 17) that undergo a structural transition upon binding of GTP, thereby amplifying the signal received. Coordination of the γ -phosphate of GTP leads to a main-chain conformational change (symbolized by the yellow arrow) at residues 84(83) and 85(84) at the N terminal of helix B, which is shifted along the amino-acid sequence (green arrow) and changes its orientation (smaller turquoise arrow). In this process, a new surface of helix B emerges which is complementary to a surface of domain 2 (yellow). As a consequence, domain 2 and 3 rotate (red arrow) and translate (large turquoise arrow) as one rigid body, to form new interfaces with domain 1. The relative magnitude of the structural changes increases from about 4 Å close to the γ -phosphate binding site to 42 Å at the tip of domain 2 and is symbolized by the relative sizes of the arrows. Green, GDP; white sphere, Mg^{2+} ion. The results of the structural transition, the rearranged structural elements as found in the GTP form of EF-Tu, are schematically shown in d.



Tyr 47, a short α -helix (A') comprising just over one turn (Fig. 2). The hydroxyl group of Tyr 47 hydrogen bonds to the α -phosphate of the GTP analogue, and Asp 51(50) (where given, numbers in brackets denote the corresponding residue in the EF-Tu from *E. coli*) at the C terminus of this short helix forms part of the secondary coordination shell of the Mg^{2+} ion, interacting with the metal ion through a water molecule. Beyond the A'

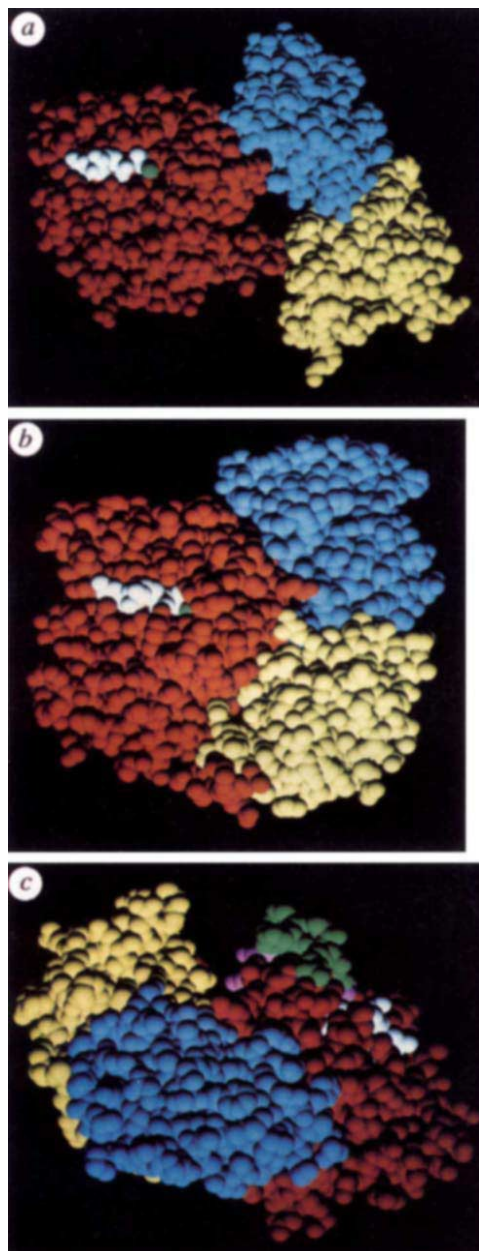


FIG. 5 Space-filling models of EF-Tu-GDP¹⁷ (a) and EF-Tu-GppNHp (b). Red, domain 1; yellow, domain 2; blue, domain 3; green, Mg^{2+} ; white, nucleotide. The orientation of the molecule is different from that in Fig. 1 but the same as in Fig. 4. Note that the hole at the centre of the molecule seen in the GDP form disappears in the GTP form, because of formation of a new interface between domains 1 and 2. c, The proposed binding site for aminoacyl-tRNA is located in a cleft between domains 1 (red) and 2 (yellow), at the top of the figure. Both the constant (green) and variant (purple) portions of the effector region (see text) contribute to formation of the cleft and may couple effector binding to hydrolysis of the nearby GTP (white). Orientation of the molecule is rotated relative to Fig. 1 to visualize the cleft. Figures prepared using Pro-Explore (Biostructure SA).

helix, the polypeptide chain changes direction at Pro 54(53) and enters another short α -helix (A'') roughly perpendicular to the first one (A') of the effector region and extending to Gly 60(59).

The effector region can be divided into two segments. The portion comprising residues 54(53) to 60(59), that is helix A'', is well conserved among prokaryotic EF-Tus. In contrast, residues in the N-terminal half of the effector region, amino acids 41–49(48), are not conserved; in fact, this is one of the most variable segments of the whole molecule. This portion of unique primary structure in a highly conserved environment may thus lend specificity to the interaction with the ribosome.

Nucleotide binding and Mg^{2+} coordination

Guanine nucleotides bind with a dissociation constant of 10^{-8} to 10^{-9} M to the EF-Tu from *T. thermophilus*²⁷. The GTP analogue, GppNHp, makes many specific hydrogen-bonding interactions with the protein (Fig. 3a, b). The guanine base is bound by interactions with two segments conserved in most GTP-binding proteins¹¹, with consensus sequences Asn-Lys-X-Asp (residues 136(135)–139(138)) and Ser-Ala-Leu/Lys (residues 174(173)–176(175)). In addition, there are hydrophobic interactions of the guanine ring system with Leu 176(175) and the side-chain methylene groups of Lys 137(136) (Fig. 3a). The ϵ -amino group of the latter side chain interacts with O4' of the ribose, whereas both O2' and O3' are exposed to solvent and make no hydrogen bonds with the protein. The phosphates of the GTP analogue are held in position by hydrogen-bonding interactions with the side chains of Lys 24, Thr 25, Thr 26, Tyr 47 and Thr 62(61), as well as with the backbone amides of the glycine-rich loop between β -strand a and the A helix (Fig. 3b).

Mg^{2+} is an essential cofactor in the GTPase reaction of all guanine nucleotide binding proteins²⁸. In this structure, a magnesium ion is coordinated to one oxygen each of the β - and γ -phosphates as well as to the hydroxyl groups of Thr 25 and Thr 62(61). The octahedral coordination sphere is completed by two water molecules which, in turn, are hydrogen bonded to the carboxylates of aspartic acid residues 51(50) and 81(80), respectively. This coordination mode is similar to that in the complex of p21^{ras} with Mg^{2+} -GppNHp¹². Comparison with the diphosphate form of the EF-Tu from *E. coli* is hampered by the moderate resolution of that structure¹⁷, which precluded the assignment of water molecules, and the fact that Asp 51(50) belongs to the segment removed by proteolysis.

GTPase mechanism

GTP hydrolysis catalysed by EF-Tu occurs by an in-line, direct attack on the γ -phosphate by a water molecule²⁹. In the electron density, there is a clearly defined, isolated water molecule (411) located 3.40 Å from the P atom of the γ -phosphate, with a hydrogen bond to one of its oxygen atoms (Fig. 3c). This water molecule is shielded from bulk solvent by the lipophilic side chains of Val 20 and Ile 61(60), thus increasing its nucleophilicity. It is also held in position by hydrogen bonding exclusively with main-chain atoms, that is, the NH groups of Gly 84(83) and His 85(84) as well as with the CO moiety of Thr 62(61). There is no interaction of this water molecule with any side chain, in particular not with those of His 85(84) and Arg 59(58), both of which have been proposed to participate in activation of the water and/or transition state stabilization^{30,31}. The guanidinium group of Arg 59(58), 8.0 Å from the water molecule, forms a strong ion pair with Asp 87(86) (Fig. 3c). The side chain of His 85(84) is exposed to solvent in the present structure. A simple rotation over its C_{α} - C_{β} bond could place the imidazole close to the buried water molecule (411), but it would have to pass the narrow 'hydrophobic gate' formed by the side chains of Val 20 and Ile 61(60) (Fig. 3c). These two residues would have to move considerably to allow the His 85(84) side chain access to the active site. Such a major movement is unlikely during the intrinsic GTPase reaction, because the importance of the proper position of the Val 20 side chain has been shown by

TABLE 1 Results of the rotation and translation searches for domains 1 and 2/3 of EF-Tu

Rotation function	Θ_1	Θ_2	Θ_3	Height/r.m.s.	Highest false peak
Domain 1	204.96	17.06	274.11	4.5 σ	1.5 σ
Domain 2/3	117.82	59.69	172.18	4.5 σ	1.7 σ
Translation function	Fractional translations			Height/r.m.s.	Highest false peak
	x	y	z		
Domain 1	0.291		0.304	7.17 σ	2.98 σ
Domain 2/3	0.182		0.439	7.59 σ	3.03 σ
Relative y-translation		0.290		3.76 σ	1.21 σ

For molecular replacement, data in the resolution range of 15 to 4.5 Å were used. Attempts at solving the rotation function using the coordinates for trypsinized EF-Tu-GDP from *E. coli*¹⁷ failed. The coordinates for each domain 1 and domain 2/3 were then used separately in molecular replacement calculations using X-PLOR⁴¹. In the search models, all side chains of *E. coli* EF-Tu that are not conserved in the *T. thermophilus* protein were truncated at the C β atom. Furthermore, because of suspected structural differences between the GDP-bound and the GTP-bound form of domain 1, the search model for this entity was restricted to segments 10–36, 65–82, 99–163, 168–178 and 186–198 (*E. coli* numbering) so that it contained only about half the atoms of the domain. As a consequence, the classic rotation function could not be solved, in spite of Patterson correlation refinement⁴¹ of the obtained cross-rotation peaks and wide variations of resolution and integration radius. The problem could finally be solved by application of the direct rotation function as proposed by Brünger⁴², that is, explicit rotation of the search molecule in 10° steps and computation as well as refinement of the Patterson correlation at each sampled orientation. The rotation function for domain 2/3 was solved using the same approach. The translation function as implemented in X-PLOR⁴¹ gave quite different results for domain 1 and domain 2/3. To combine the two independent solutions of the x, z translation function, the x, z solution for domain 2/3 was translated along the y-axis while coordinates for domain 1 were held fixed, until a maximum of the translation function correlation was obtained.

the strong reduction of (intrinsic) GTPase activity upon replacement of this residue by glycine and the concomitant increase in the nucleotide exchange rate³².

But this kind of rearrangement could be involved in the dramatic increase of the GTPase activity of EF-Tu upon binding of the ribosome to the effector region, residues 41–62. This might break the strong ion-pair between Arg 59(58) and Asp 87(86); the latter might reorient towards His 85(84) which is then pushed through the 'hydrophobic gate' formed by Val 20 and Ile 61(60), towards the active-site water molecule where it acts as a general base. Such a mechanism is possible because Ile 61(60), one of the two 'wings' of the gate, is part of the effector region and may therefore be moved out of the way upon effector binding.

The conformational 'switch' in the G domain

When GDP is exchanged for GTP, the protein has to provide additional ligands to stabilize the γ -phosphate and the active-site water molecule predestined to attack that γ -phosphate in the hydrolysis reaction. EF-Tu does so by changing the conformation of Gly 84(83), the amide of which is hydrogen bonded to the carboxylate of Asp 87(86) in the GDP form¹⁷. This latter hydrogen bond is broken, and the Gly 84(83) amide serves as a ligand to both the γ -phosphate group and the nucleophilic water (411). Similarly, the peptide chain conformation at His 85(84), α -helical in the GDP form, changes (to a β -conformation) to orient its main-chain amide towards this water molecule, so that the NH groups of residues 84(83) and 85(84) act as a bidentate ligand (Fig. 3c). Asp 87(86), now devoid of the strong hydrogen-bonding interaction of its side chain with Gly 84(83) because of the conformational change of the latter, finds a new partner in Arg 59(58) and forms an ion pair with this residue. This in turn moves the side chain of Tyr 88(87), which in the GDP form is buried between strands a and c of the domain 1 β -sheet, towards the surface of the domain, leading to a difference in the position of its phenolic OH group as large as 13.4 Å (Fig. 4a, b).

In the GDP form of the EF-Tu from *E. coli*¹⁷, helix B extends from His 85(84) to Thr 94(93). The drastic conformational changes experienced by its N-terminal residues (His 85(84) to Tyr 88(87)) upon GTP binding preclude the start of the α -helix at His 85(84) in this structure. Residues 86(85) to 88(87) form a β -turn instead, and the polypeptide chain enters an α -helix only at Ile 89(Val 88). Thus, the start of the B helix is delayed by as much as four residues relative to the GDP form. Moreover, it extends further by two residues, having its C terminus at Ala 96(95) instead of Thr 94(93).

Signal amplification and domain rearrangement

Not only is the B helix shifted along the polypeptide chain in the triphosphate form of EF-Tu discussed here, it also has an orientation very different from the GDP form (Fig. 4a, b). As a result, the C-terminal residues of this helix have positional differences of up to 7.5 Å between the two structures. This illustrates the amplification of the signal (GTP binding) on its way through the protein molecule (Fig. 4c). The transition from guanosine diphosphate to triphosphate is small, as is the immediate consequence, the main-chain conformational changes at Gly 84(83) and His 85(84), which lead to differences in their NH positions of just 4.6 Å and 3.6 Å, respectively. But the consequences of these minor rearrangements are already larger. Both Asp 87(86) and Tyr 88(87) undergo major reorientations of their side chains (see above), and the B helix is shifted along the sequence and changes its orientation. As a result, there is a steady increase in atomic deviations along the length of the helix, culminating at alanines 96(95) and 97(96). Furthermore, both the reorientation of the B helix and its C-terminal extension relative to the GDP form change the surface of this portion of domain 1. In the GTP form, this surface complements a surface of domain 2. Domain 2 is now attracted and leaves the position into which it was pushed by repulsion from a non-complementary surface of domain 1 in its diphosphate form. It forms a new, tight interface with that domain, making the hole separating domains 1 and 2 in the GDP conformation of EF-Tu¹⁷ disappear in the triphosphate structure (Fig. 5a, b). By formation of this new interface, the signal is transmitted into domain 2, where it leads to positional differences for individual amino acids of up to 42 Å (for the C α atom of residue 232(221)). Thus, over a distance of 29 Å from the binding site for the γ -phosphate, the signal is amplified so much that it translates into a huge structural rearrangement of whole domains (Fig. 4c, d).

The new interface between domains 1 and 2 is mainly formed by polar side chains which form five salt bridges and eight hydrogen bonds (Fig. 4b). Almost all the residues involved interact exclusively with solvent in the GDP form of *E. coli* EF-Tu¹⁷ (Fig. 4a). These polar interactions between side chains are more directional than, for example, hydrophobic forces, leading to greater specificity, and they can be disrupted without interfering with the fold of the main chain of the individual domains. As a result, both types of the structure, interface closed (triggered by GTP binding) and interface open (triggered by GDP binding), are biologically competent, which would probably not be the

case were the interface hydrophobic. The domain 1/2 interface is thus designed to be broken and specifically reformed, depending on the signal received.

Domain 3 moves along with domain 2 and forms a largely polar interface with domain 1, mainly involving side chains of the C helix of the latter. Although domains 1 and 3 do have contacts in the GDP form¹⁷ (Fig. 4a), the residues involved are different from those in the present structure (Fig. 4b). This is due to the reorientation of domain 3 and of the domain 1 B helix, the N-terminal residues of which are responsible for a large portion of the interaction with domain 3 in the GDP form¹⁷.

The interface between domains 2 and 3 is partly hydrophobic and well preserved during the huge rigid body movement of domains 2/3 initiated by GTP binding. As a result, the Ca atoms of domains 2/3 of our structure, when rotated and translated over the structure of the GDP form, show a r.m.s. difference of only 1.1 Å (for 160 equivalent residues).

Aminoacyl-tRNA binding to the EF-Tu

EF-Tu-GTP, but not EF-Tu-GDP, binds aminoacyl-tRNA during the protein elongation cycle. The binding site for aminoacyl-tRNA on EF-Tu has not been identified before. Crosslinking^{33,34} and photo-oxidation studies³⁵, and partial proteolytic cleavage of *E. coli* EF-Tu³⁶ suggested that His 67(66) and residues 47(46)–59(58) are near the aminoacyl-residue binding site. Although these residues are located in domain 1, the isolated domain 1 of *E. coli* EF-Tu does not form complexes with aminoacyl-tRNA³⁷. More recent crosslinking studies implicated an interaction of domains 2 and 3 of eukaryotic EF-1α with aminoacyl-tRNA³⁸. But no interaction of aminoacyl-tRNA with the isolated domains 2/3 of EF-Tu from *T. thermophilus*

was detectable³⁹. These observations suggest that all three domains are involved in tRNA binding, with the aminoacyl end interacting with domain 1, close to the C-terminal part of the effector region.

We propose that the binding of the aminoacyl-tRNA occurs in a solvent-filled cleft formed between domains 1 and 2 of EF-Tu-GTP (Fig. 5c). This cleft is lined with several positively charged side-chains that should interact with the phosphates of the tRNA. These residues include Lys 45 and Lys 52 of the effector region, Lys 2, Lys 90(89), His 273, Lys 275(263), Arg 295(283) and Arg 300(288). Arg 59(58) is at the rim of the cleft. Recent ¹³C-NMR experiments showed that replacement of this residue by Thr altered the interaction of aminoacyl-tRNA with EF-Tu (C. Förster, S. Limmer and M.S., unpublished results). At the very bottom of the cleft is His 67(66), the target of one of the aminoacyl-tRNA crosslinking experiments mentioned before. This side chain was also found to be protected against photo-oxidation in the presence but not in the absence of aminoacyl-tRNA³⁵. Because the presence of aminoacyl-tRNA is necessary for physiological, ribosome-induced GTP hydrolysis, it is reasonable to expect the tRNA-binding site close to the effector region and to the GTP binding site. Figure 5c shows that the proposed binding cleft is indeed in their vicinity.

This model also explains the necessity of the switch from EF-Tu-GDP to EF-Tu-GTP for aminoacyl-tRNA binding, because the cleft is only formed upon domain rearrangement induced by a conformational change of Gly 84(83). Replacement of this residue by Ala should prevent the structural switch and the formation of the cleft, and aminoacyl-tRNA binding to this mutated EF-Tu should not occur. This is what has been observed⁴⁰, thus further supporting our assignment of the binding site for aminoacyl-tRNA on active elongation factor Tu. □

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Internal structure and polarization of the optical jet of the quasar 3C273

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THE extragalactic radio source 3C273 was the first quasar to be identified¹, and remains one of the nearest and most luminous quasars known. In radio images²⁻⁴, it appears as a bright, point-like nucleus from which emerges a single large jet; the jet is also apparent in optical images⁵⁻⁸, but the relatively poor resolution of ground-based optical data has hampered detailed comparisons with the radio maps. Here we present high-resolution intensity and polarization maps of the optical jet of 3C273 obtained using the Faint Object Camera on the Hubble Space Telescope. The optical emission is tightly confined to the core of the radio jet, and is resolved into a number of highly polarized knot structures. Both the optical and radio emission can be explained by synchrotron radiation from a stream of energetic particles burrowing into the surrounding medium. Comparison with the radio maps also reveals asymmetry in the emission across the jet, indicating significant lateral motion of the jet relative to the ambient medium. The onset of optical emission from the jet some distance from the nucleus of 3C273 could arise from the interaction of the jet with a shell of gaseous material surrounding the host galaxy.

The radio emission from the jet of 3C273 has been mapped from the ground at resolutions down to 0.22 arcsec (ref. 2), and on milli-arcsec scales^{3,4} in the nucleus using very-long-baseline interferometry. The resolution of ground-based optical data, on

the other hand, is limited to typically ≥ 1 arcsec (full-width half-maximum, FWHM) by atmospheric seeing. The HST images presented here have a resolution of 0.15 arcsec (FWHM) and this has enabled the optical jet to be resolved across its width. Figure 1 shows the total intensity image of the optical jet of 3C273 taken by the Faint Object Camera (FOC) on the Hubble Space Telescope (HST), together with a recent ground-based image which includes the central quasar (not seen in the HST image owing to the small field of view of the FOC). The optical jet extends between 12 and 22 arcsec from the unresolved nucleus at a position angle of 222° in the plane of the sky. At a redshift of 0.158, the estimated distance to 3C273 is 560 Mpc ($H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$; $q_0 = \frac{1}{2}$), giving a scale of 1 arcsec to 2.5 kpc. The optical jet thus extends for more than 50 kpc (in projection) from the quasar nucleus. But if the jet is highly inclined towards our line of sight, the actual extent of the jet could be much greater. For comparison, the optical jet in the giant elliptical galaxy M87 is only 2.5 kpc (in projection), and probably less than 5 kpc (deprojected). The optical jet of the quasar 3C273 is resolved by the HST into a number of highly polarized knots, for which we have adopted and extended the labelling convention of previous ground-based imaging studies⁵⁻⁸ (see Fig. 3). The width of the optical jet is typically only a few tenths of an arcsecond. At its widest point, farthest from the quasar, the optical jet is just over half an arcsecond across. It has hitherto not been clear whether the offset feature, known as the inner extension, is actually part of the jet. This was thought likely because of its similar colour^{5,6}. The high polarization measured by the HST (see Fig. 3) supports the association of the inner extension with the jet.

Figure 2 shows a grey-scale map of the reconstructed HST image superimposed on contour plots of the 2 cm and 6 cm radio emission². The radio emission is broader than the optical emission, even after taking account of the different resolution, and reaches a maximum ~ 1 arcsec further away from the quasar

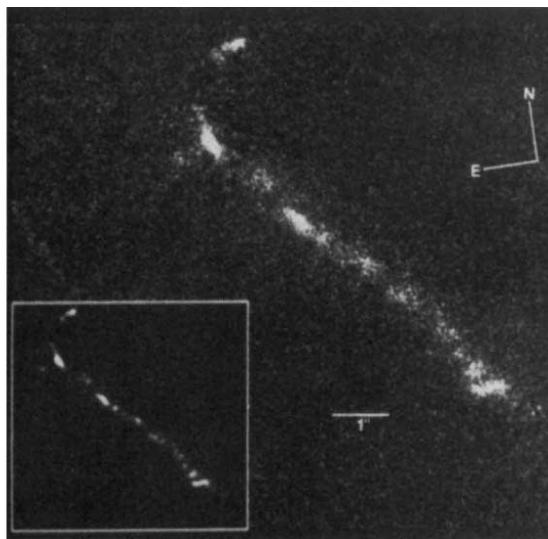
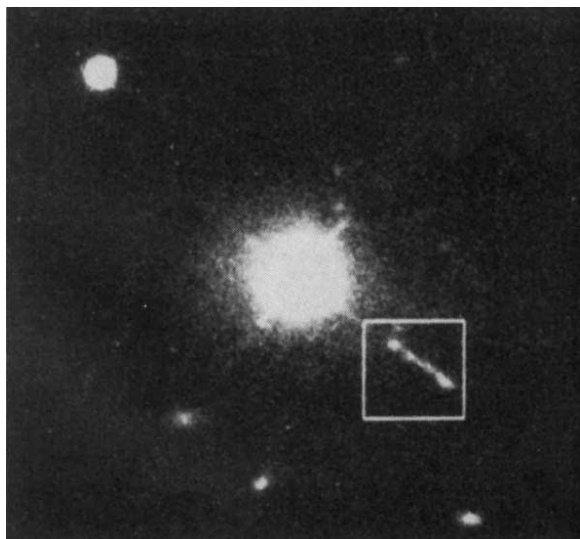


FIG. 1 Total intensity image of the optical jet of 3C273. The HST observations were made on 6–8 January 1992 using the FOC. Eighteen exposures were taken, each of approximately 1,300 s duration, using the F430W (B band) filter in conjunction with the three polarizers (POL0, POL60 and POL120) in the f/96 camera. This has a field of view 11×11 arcsec and a pixel size of 0.022×0.022 arcsec. The raw images were flat-fielded, geometrically corrected, and combined to produce a single total intensity image and a polarization map (Fig. 3) of the optical jet^{20,21}. Left is a ground-based V band image of 3C273 and the optical jet. (This image was kindly obtained for us by M. Colless and D. Schade at the Canada–France–Hawaii Telescope (CFHT) on Mauna Kea.) Right

is the B band HST image. The principal effect of the spherical aberration is to reduce the contrast of a source against the background, producing a loss in sensitivity. Image reconstruction techniques such as maximum-entropy deconvolution can help to recover some of the lost signal for data with sufficient initial signal to noise. Such techniques, do not, however, work so well with low signal-to-noise data²² and the best reconstruction possible with the HST data presented here (inset, right) has an effective resolution of 0.15 arcsec (FWHM), which is similar to the best radio maps currently available. For comparison, the ground-based CFHT image has a seeing-limited resolution of 0.7 arcsec (FWHM).

than the outermost bright optical knot (knot D3). The optical emission from the jet runs along the ridge line of the radio emission and is clearly asymmetric with respect to the fainter radio contours (Fig. 2b) which are more extended on the south-east side of the jet.

Figure 3 shows the magnetic polarization vectors measured by the HST superimposed on a contour plot of the reconstructed image. The brightest optical knots are all highly polarized (20–50%). This is much greater than either the polarization owing to interstellar extinction or that owing to the FOC itself, both of which are probably less than 1%. The magnetic polarization vectors are aligned roughly parallel to the jet nearest to the quasar (knots A1 and B1), but their orientation changes significantly along the length of the jet. At the outermost point (knot D3), the magnetic polarization vectors have twisted around by 40° to become parallel with the inclined linear structure of knot D3. The twisting of the polarization vectors at the position of knot D3 can also be seen in ground-based optical polarization measurements^{9,12}. The inner extension is also found to be highly polarized (~ 10 – 20%) and the polarization position angle is not aligned with the main jet. The high polarization supports its association with the jet, although its misalignment is puzzling.

These results confirm the high polarization of knot A1, as reported by Scarrott and Warren-Smith¹⁰ and by Scarrott and Rolph¹¹, but are inconsistent with the low polarization reported by Röser and Meisenheimer^{9,12}. The requirement^{9,12} for an additional unpolarized thermal contribution is not supported by the HST data, which are consistent with pure synchrotron radiation for the optical as well as the radio emission. In this model, the

polarization depends on the relative strengths of the unresolved (assumed random) magnetic-field component (B_{ran}) and resolved (uniform) field component (B_{uni}), and also on the inclination angle and velocity of the jet. If the jet is non-relativistic, then the observed high polarizations ($p \approx 0.3$) imply that either $B_{\text{ran}} \ll B_{\text{uni}}$ with the jet aligned nearly parallel to the line of sight, or $B_{\text{ran}} \approx B_{\text{uni}}$ with the jet nearly perpendicular to the line of sight. If the jet is relativistically beamed toward us, however, then the dependence of the polarization on the inclination angle is counteracted by the relativistic aberration and the observed polarizations imply that $B_{\text{ran}} \approx B_{\text{uni}}$.

The polarization map is not consistent with the suggestion that the optical emission is quasar light scattered in our direction by jet particles¹³. If this were the case, then (like all reflection nebulae) the position angle of the magnetic polarization vector would be everywhere parallel to the radius vector to the quasar. But the data show significant deviations from the expected position angle. The emission mechanism is most likely synchrotron radiation from relativistic electrons accelerated by the magnetic field of the jet. Assuming a reasonable (minimum-energy) magnetic-flux density in the jet of $20 \text{ nT}^{9,12}$, the estimated lifetime of the high-energy optically emitting particles is very short ($\sim 100 \text{ yr}$) compared to the longer lifetime of the less-energetic radio-emitting particles ($\sim 30,000 \text{ yr}$). The radio-emitting particles can thus diffuse farther from their site of origin than the shorter-lived optically emitting particles. It is therefore not surprising to see that the optical emission from the jet of 3C273 is more confined to the core of the jet than is the radio emission.

We interpret these data in terms of a continuous fluid model

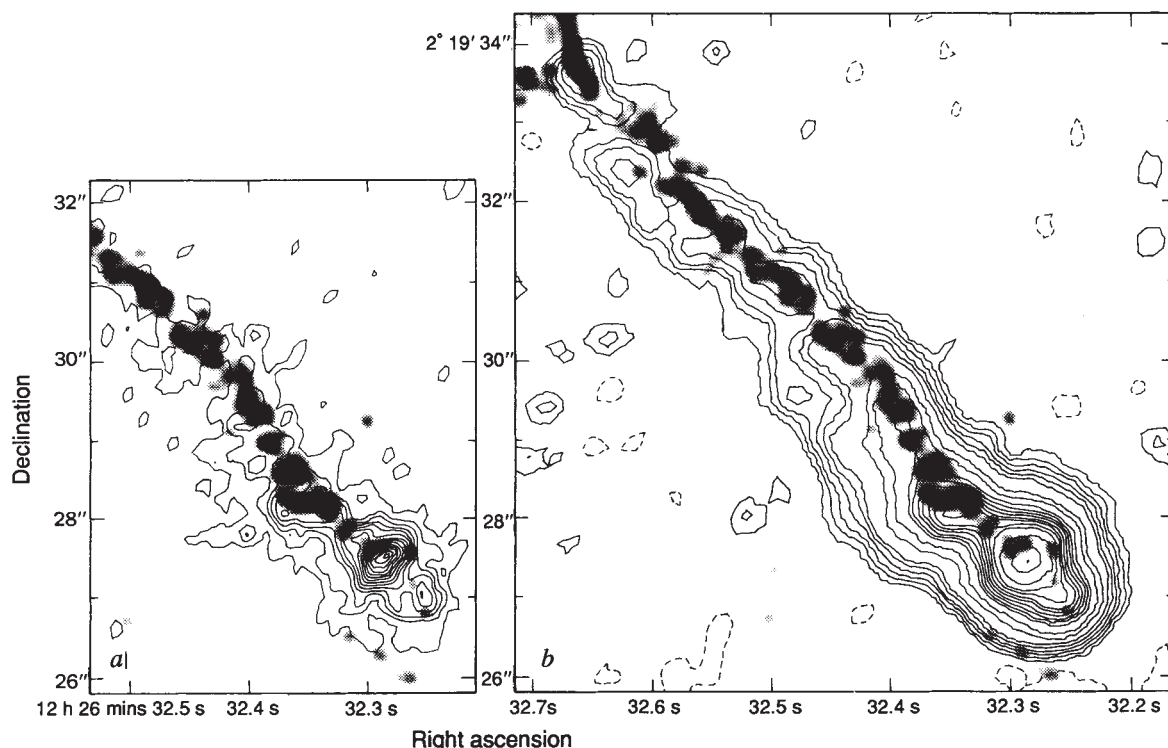


FIG. 2 Reconstructed HST image of the optical jet of 3C273 (grey scale) superimposed on a, the 2 cm and b, the 6 cm VLA radio maps (contours) of the jet². The resolution of the HST image is 0.15 arcsec (FWHM), and the resolution of the 2 cm and 6 cm VLA radio maps are 0.22 arcsec (FWHM) and 0.35 arcsec (FWHM) respectively. The HST image has been aligned with the radio image to within 0.1 arcsec accuracy. This was achieved in two stages. First the CFHT image was calibrated using accurate positions (better than 0.1 arcsec) of reference stars¹⁹ which appear in the full image (not shown). Accurate positions of the well

defined knots A, B, D and the inner extension of the optical jet could then be determined using the calibrated CFHT image. Second, the reconstructed HST image was blurred to a resolution comparable with the CFHT image (that is 0.7 arcsec FWHM). The positions of the knot centroids determined from the CFHT image were then used to calibrate the reconstructed HST image. Before comparing with the radio data, the resultant positions were translated to make the optical quasar position coincide with the radio position given in the VLA Calibrator List [RA 12 h 26 m 33.248 s, Dec. $+02^\circ 19' 43''.29$ (1950.0)].

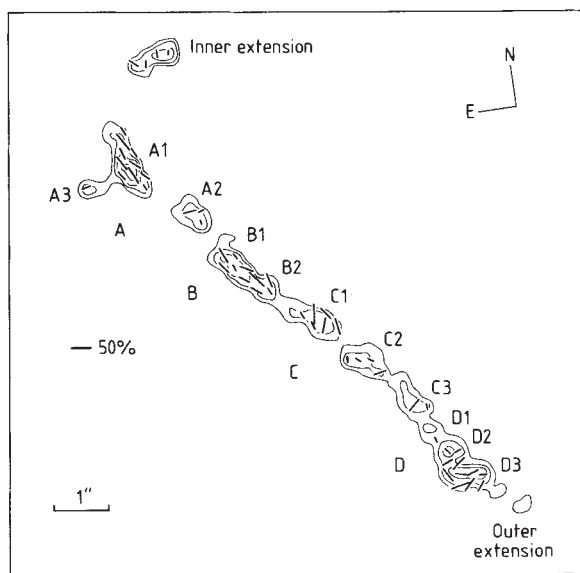


FIG. 3 Polarization map of the optical jet of 3C273 superimposed on a contour plot of the reconstructed HST image. The map shows the orientation of the magnetic-field vectors. For synchrotron radiation, this is the direction of the projected magnetic field in the emitting region. The effective resolution of the polarization map is 0.15 arcsec and the data have been binned over 8×8 pixels. The principal source of error in these measurements is the in-orbit calibration of the FOC polarizers. Improved calibration will most probably affect the position angle offset which could be in error by up to 10° , although it is likely to be much less than this. Note that a recalibration of the position angle offset would rotate all polarization vectors by the same amount. The relative error in the polarization measurements are estimated to be $\leq 10\%$ and the corresponding position angle error is $\leq 3^\circ$. The labelling convention adopted for the knots and extensions in the jet is also shown.

in which a jet of ionized plasma flows out from the quasar into the surrounding medium. The large swing in position angle of the polarized emission at knot D3 probably delineates the termination of the jet where it hits the end of the cavity that it has burrowed in the surrounding medium. The curvature of the jet in the vicinity of knot C and the flattened morphology of knot D3 are similar to the structures that form in non-axisymmetric simulations of precessing jets which are not fixed with respect to the surrounding medium¹⁴, and also in simulations of jets exposed to a cross wind¹⁵. In either case, the distinguishing feature of these models is the lateral motion of the jet relative to the ambient medium. A north westerly cross wind with a velocity of 300 km s^{-1} and a density of 10^{-3} cm^{-3} in the vicinity of 3C273 could bend the jet²³ as observed. Detailed numerical simulations^{14,15} produce structures similar to knot D and the radio hotspot at the end of such a jet. The asymmetry of the longer wavelength radio emission can also be explained by the action of such a cross wind which would direct the backflow from the jet into the low frequency radio lobe²⁴ on the south-east side. The higher density and compressed magnetic field of the ambient medium on the upwind side of the jet could also explain the higher rotation measure found on the north-east side of the jet.

The sudden onset of optical emission at knot A1 marks the point where the loss-free progress of the jet from the nucleus of 3C273 stops and optical radiation losses becomes significant. We suggest that this is due to the interaction of the jet with a shell of gaseous material which surrounds 3C273, for which there is some evidence in the ground-based Canada–France–Hawaii

Telescope image (Fig. 1). A faint arc intersects the jet at the same radius as knot A1. The inner extension forms part of this arc, as does the fainter extension of the other side of the jet (knot A3).

Such a model has been suggested previously to explain the formation of structure in the jet of the nearby active galaxy Cen A¹⁶, where a rich and complex system of shells is known¹⁷ to exist. Some of the brightest knots in the radio emission from Cen A appear just where the jet crosses these shells¹⁶. These shells were probably formed during a collision with another galaxy¹⁸. Gas-rich material captured by Cen A during the collision now forms the conspicuous warped dust lane which encircles the galaxy and probably provides the fuel that powers the radio emission via an obscured central engine. Such shells are also likely to form around quasars, either by interaction with another galaxy, or by the intense wind from the central quasar which sweeps up the interstellar medium in the host elliptical galaxy¹⁹.

If this interpretation is correct and the shell feature is real and edge-brightened, then the jet in 3C273 cannot be aligned nearly parallel to the line of sight; it must be viewed in the plane of the sky nearly perpendicular to the line of sight. This follows from the observed coincidence of knot A1 with the (possibly) edge-brightened shell, which would then be a remarkable coincidence if the jet were actually aligned nearly parallel to the line of sight. Consequently, the optical jet in 3C273 could not be beamed toward us and must be one-sided. Additional data are required to test this model, particularly very deep high-resolution imaging of the region around knot A. □

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Identification of PSR1758–23 as a runaway pulsar from the supernova remnant W28

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THE discovery¹ of the pulsar 1758–23 in the vicinity of the radio supernova remnant W28 has prompted speculation that the two might be physically associated. Although the brightening of the supernova remnant in the direction of the pulsar suggests such an association², the large column density of electrons in the interstellar medium towards PSR1758–23 (evident from the pronounced dispersion of the radio signal) and the anomalously large scattering observed in the pulses seem to indicate^{1,3} a distance much greater than that to W28. Here we present new observations which indicate that the large dispersion and scattering of the pulses is instead caused by a dense screen of ionized material located along the line of sight, and thus the distances to the pulsar and W28 can be reconciled. Similar scattering screens have been reported previously^{4,5} and appear to be associated with H II regions; the properties of these screens seem, however, to be inconsistent with current models⁶ of turbulent structure in the interstellar medium.

Observations were conducted at the Very Large Array (VLA) to determine the location of the pulsar with respect to W28 and to constrain the distance to the pulsar by means of 21-cm H I absorption spectroscopy. A VLA A-array image at 1.46 GHz (resolution 1 arcsec) revealed two sources with flux densities of 22 mJy and 7 mJy within the $\pm 4'$ pulsar error circle¹. As the latter source exhibited 8% linear polarization, it was thought to be the pulsar. A successful search for pulsations at 1.46 GHz (14 December 1992) and 1.67 GHz (4 January 1993) confirmed this assumption. The properties of the pulsar are summarized in Table 1.

The pulsar clearly lies outside the radio boundaries of W28. Nonetheless, the circumstantial evidence presented earlier² and the recently determined young³ age for the pulsar, $\tau_c = 5.8 \times 10^4$ yr (the so-called characteristic age, $P/2\dot{P}$) remain supportive of the proposed association. In the context of the model

discussed in ref. 2, the pulsar has apparently overtaken the decelerating blast wave of the supernova remnant (SNR).

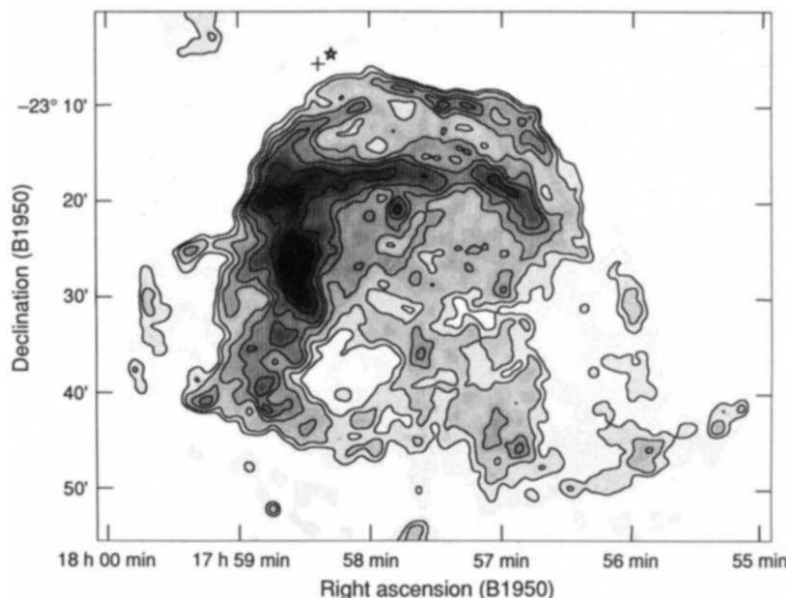
The 21-cm H I absorption spectra towards the pulsar and 1757–231 (an extragalactic radio source in the same field as shown in Fig. 1), are shown in Fig. 2. These data are compared with the H I absorption spectra towards W28, M8 (ref. 7) and other objects in that area of the sky, including PSR1800–21 ($DM = 234 \text{ cm}^{-3} \text{ pc}$, $d = 4.5 \text{ kpc}$ (ref. 8)) and an extragalactic radio source⁹ 1759–225; here DM is the dispersion measure—the column density of interstellar electrons between us and the pulsar, measured in the usual units of $\text{cm}^{-3} \text{ pc}$.

The absorption profiles of W28 (ref. 7) and PSR1758–23 are similar: absorption is detectable from 0 km s^{-1} to $+23 \text{ km s}^{-1}$ with discrete absorption features at $V_{\text{LSR}} = +7.3 \text{ km s}^{-1}$ and $+17.6 \text{ km s}^{-1}$ (V_{LSR} refers to the velocity with respect to the Local Standard of Rest). None of the Galactic sources have absorption at negative velocities. The velocity width of the absorption for W28 and PSR1758–23 is 20 km s^{-1} . In contrast, the two extragalactic sources have deep absorption all through the velocity range of bright H I emission, from -30 km s^{-1} to at least $+23 \text{ km s}^{-1}$, and an absorption width of 80 km s^{-1} . The absorption width gives an approximate measure of relative distances, and so the difference in absorption widths between PSR1758–23 and the extragalactic radio sources rules out the pulsar being located at 13.8 kpc (the distance estimated from a recent interstellar electron-distribution model¹⁰). The similarity of their absorption profiles is such that it is likely that W28 and PSR1758–23 are at a similar, if not the same, distance.

Several estimates of distance (for example, ref. 2) to W28 based on the Σ - D (surface brightness–diameter) relation exist in the literature. Given the (well-founded) criticisms of this relation¹¹ we ignore such estimates. A kinematic distance can be estimated from either the mean expansion velocity of H α filaments¹² ($V_{\text{LSR}} = 18 \pm 5 \text{ km s}^{-1}$) or H I absorption⁷ ($V_{\text{LSR}} = 17.6 \pm 0.3 \text{ km s}^{-1}$). We note that in this direction there is also strong CO emission near these velocities, suggesting that W28 is expanding into dense molecular material¹³. Assuming a flat rotation curve and an uncertainty of $\pm 7 \text{ km s}^{-1}$, to allow for deviations from circular rotation¹⁴, the distance, d , must be between 2.5 kpc and 4.2 kpc. Lower distance estimates are preferred by those who associate W28 with the H II regions M8 and M20, whose distances are estimated¹⁴ to be about 1.8 kpc. Given this uncertainty we adopt a distance to W28 of $3d_3$ kpc (where $d_3 = 1/3$ kpc).

A proper understanding of the scattering screen, in particular its location and physical parameters (density and temperature)

FIG. 1 A radio image of the W28 region at 327 MHz made with the VLA from unpublished data of D.A.F., N. E. Kassim and K. W. Weiler. Contours are at 25, 50, 100, 200, 300, 400, 500 mJy beam⁻¹, with the image smoothed to a resolution of $65''$. The r.m.s. noise in the image is $\sim 8 \text{ mJy beam}^{-1}$. The position of PSR1758–23 and the extragalactic source 1757–231 are indicated by the five-point star and the cross, respectively.



are needed before the proposed pulsar-SNR association can be considered probable. The large dispersion measure and the large scattering tail indicate substantial foreground ionized material which must also be capable of significant scattering. Let n_e be the true density, R be the thickness of this screen and ϕ the filling factor (that is, the fraction of the line of sight occupied by the ionized gas of density n_e). Constraints on these parameters are provided by the observed dispersion measure, $DM = \int n_e dl = n_e R \phi$ and an upper limit to the emission measure (EM) in this direction, $EM = \int n_e^2 dl = n_e^2 R \phi$.

A stringent upper limit to this emission measure can be obtained by attributing the observed radio continuum emission to free-free emission from ionized gas after subtracting the expected non-thermal background contribution from relativistic electrons spiralling in the magnetic field of our Galaxy. In this direction¹⁵, at frequency $\nu = 10$ GHz, $T_B(10 \text{ GHz}) = 0.25 \text{ K}$ (T_B = brightness temperature). By subtracting the expected synchrotron component of the emission, $T_B(0.408) (10 \text{ GHz}/0.408 \text{ GHz})^{-2.7}$ (where $T_B(0.408) = 7 \times 10^2 \text{ K}$ at the pulsar position seen in the Molonglo 0.408 GHz map¹⁶ of the Galactic plane) we find $EM = 4.5 \times 10^3 T_8^{-0.35} \text{ cm}^{-6} \text{ pc}$; here $T_8 = (T_e/8,000) \text{ K}$ where T_e is the temperature of the ionized region and T_8 is usually assumed to be unity. The lowest reliable detection in an H85 α recombination

TABLE 1 Properties of PSR1758-23

Right ascension (B1950)	17 h 58 min 17.572 s (± 0.009)
Declination (B1950)	-23° 04' 43.4" (± 0.2)
Pulse period (ms)	415.7789 $\pm$ 0.0001
Epoch (MJD)	48,991.8
Period derivative ($10^{-15} \text{ s s}^{-1}$)*	112.983 $\pm$ 0.003
Characteristic age (10^3 yr)*	58
Dispersion measure (pc cm^{-3})	1,063 $\pm$ 7
Flux density at 1.46 GHz (mJy)	6.7 $\pm$ 1.3
Temporal broadening at 1.67 GHz (s)	0.07

* From Kaspi *et al.*³.

line survey¹⁷ of the W28 region is $3.7 \times 10^3 \text{ cm}^{-6} \text{ pc}$. Consequently we adopt $E = 3 \times 10^3 E_3 \text{ cm}^{-6} \text{ pc}$ as a plausible (upper limit) value for EM. This choice is compatible with $EM = (1,400 \pm 400) T_8^{-1.35} \text{ cm}^{-6} \text{ pc}$ derived from the measurement¹⁸ of the free-free optical depth towards W28.

In models for the distribution of diffuse electrons in the Galaxy¹⁰, the dispersion measure to a pulsar located at 3 kpc is $\sim 130 \text{ cm}^{-3} \text{ pc}$. Thus the excessive dispersion measure attributable to this ionized region is $D_x \sim 10^3 \text{ cm}^{-3} \text{ pc}$. It follows that the parameters of the region are $n_e = E/D_x = 3E_3 \text{ cm}^{-3}$ and $R = 333\phi^{-1} \text{ pc}$, values typical of the extended, low-density envelopes surrounding classical H II regions¹⁹. We now need to demonstrate that these parameters for the scattering screen are also consistent with the observed unusually large scattering.

Fluctuations in the interstellar plasma density, δn_e , cause multipath propagation which result in broadening of source images and give rise to an exponential tail in pulsar pulse profiles. The standard scattering theory⁶ assumes a Kolmogorov distribution for δn_e , a power law power spectrum with an exponent of 11/3 and an inner and outer length scale for the density irregularities. The e -folding timescale of the pulse profile for PSR1758-23, is $\tau = 0.07 \text{ s}$ at 1.67 GHz (Table 1). Thus at 1.46 GHz, the predicted size of the pulsar (full width at half maximum) is $\theta_p = 0.42'' (d_3 f)^{-0.5}$ and that for any extragalactic source $\theta_E = \theta_p (1+f)$; here f is the ratio of the observer-screen distance to the pulsar-screen distance. In our 1.46 GHz image, the pulsar and the extragalactic source 1758-231 are indistinguishable from the beam, implying $\theta \leq 1''$ for both. Solving the above relations for the unknown geometrical term f we find $0.3 \leq f \leq 3.4$. The pulsar and the scattering material cannot be co-located. Thus the same H II region envelope that is responsible for D_x is also the likely source of the scattering in the pulse profile for PSR1758-23.

The unusually large observed scattering tail requires that the screen has some unusual parameters (regardless of the distance at which the pulsar is located). The plasma density fluctuations δn_e will contribute to the free-free emission along the line of sight to PSR1758-23 and W28. We estimate this contribution, $\int \langle \delta n_e^2 \rangle dl$, obtained from an integration of the Kolmogorov irregularity spectrum on all spatial scales between the inner l_i and outer l_o scales. Following ref. 20 and using θ_E , we can infer a minimum emission measure in the screen, $EM_{SM} = 1.4 \times 10^5 l_o^{2.3} l_i^{1.3} (1+F^2) \text{ cm}^{-6} \text{ pc}$, where $F = n_e/\delta n_e$ and l_o the outer scale in parsec, and l_i the inner scale in units of 100 km, are usually assumed to be unity⁶. Even the minimum value of EM_{SM} ($F=1$) is in conflict with our measured limit on E . Equality of E and EM_{SM} , demanded by our observations, requires that $l_o \leq 10^{-2} \text{ pc}$. The small outer scale is certainly unusual⁶ and implies that the energy introduced into the turbulent cascade is at a scale much smaller than that commonly assumed. This small outer scale provides an important new constraint in identifying the agent(s) responsible for plasma turbulence in the interstellar medium. We wish to draw attention to a similar pattern towards other heavily scattered lines of sight^{4,5} and suggest that this may be a general property of the density irregularity spectrum along lines of sight with enhanced scattering.

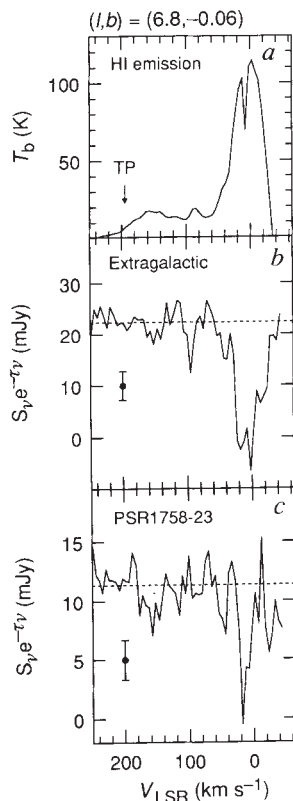


FIG. 2 H I spectra towards PSR1758-23 and a field extragalactic source (1758-231), obtained using the VLA (14 January, 1993). The correlator was gated with a duty cycle of 34%, taking data only while the pulsar was 'on'; the latter signal was derived from the output of the phased VLA (see ref. 8 for details). A bandwidth of 1.56 MHz and 64 frequency channels were used for a resolution of 24.4 kHz or 5.2 km s^{-1} . The emission spectrum (a) towards PSR1758-23 was obtained by summing the total power spectra from all 27 VLA antennas with primary beam of $30'$. The absorption spectra towards 1758-231 and the pulsar are shown in b and c respectively. The mean continuum flux for each is 22.4 mJy and 11.3 mJy (gated), and is shown by the dotted lines. The tangent point (TP), defined as the maximum radial velocity allowable by circular rotation, is indicated. Error bar indicates the r.m.s. noise determined away from the regions of absorption.

We have argued above that PSR1758–23 and W28 are located at similar distances, and have similar ages. None of the data are in conflict with the pulsar being located at the distance of W28, 3 kpc. The unusually strong scattering screen, most probably an H II region or its envelope, is constrained to lie between us and the pulsar. This screen accounts for the excessive dispersion measure and the observed scattering tail of the pulsar profile. The reasons initially used² to argue for an association (the bilateral symmetry and the bi-hemispherical asymmetry) remain strong. The high polarization and flat spectral index of W28 are reminiscent of another old plerionic SNR, G114.3+0.3 (ref. 21), which contains a pulsar of an age similar to that PSR1758–23. For all these reasons we suggest that PSR1758–23 is the run-away pulsar of W28.

Several predictions follow from our assertion. A proper motion of $20d_3^{-1}$ mas yr⁻¹ corresponding to a transverse speed of $290d_3$ km s⁻¹ (directed away from the remnant) is predicted². Careful multi-frequency imaging of the radio shell of W28 should reveal steepening of the spectral index away from the pulsar. Angular broadening measurements of the pulsar, the extragalactic source 1758–231 and an OH (1,720 MHz) maser in the molecular material proximate to W28 (ref. 22) will determine f . The same measurements will help in independently verifying our claim of a small l_0 .

This new association shows the crucial role played by pulsars in increasing the detectable life of radio remnants, either by maintaining a high surface brightness (as in G114.3+0.3, ref. 21) or rejuvenating a portion of the remnant (as in CTB80 and G5.4–1.2; refs 23 and 24, respectively). The radio properties of W28 (flat spectral index, high polarization) attest to the import-

ance of pulsar activity on the radio evolution of old remnants, an area that sorely needs some theoretical attention. □

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A physical system with qualitatively uncertain dynamics

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THE notion of determinism in classical dynamics has been eroded since Poincaré's work¹ led to the recognition that dynamical systems can exhibit chaotic behaviour, in which small perturbations grow exponentially fast. For a chaotic system, ubiquitous measurement errors, noise and computer round-off severely limit the time over which, given a precisely defined initial state, one can predict the detailed subsequent evolution. Practically speaking, the behaviour of such systems is quantitatively non-deterministic. Nevertheless, as the state of the system tends to be confined to an 'attractor' in phase space, at least its qualitative behaviour is predictable. Another challenge to determinism arises, however, when a system has competing attractors towards which an initial state may be drawn. Perturbations make it difficult to determine the fate of the system near the boundary between sets of initial conditions (basins) drawn toward different attractors, particularly if the boundary is geometrically convoluted². Recently, mathematical mappings were found³ for which the entire basin of a given attractor is riddled with 'holes' leading to a competing attractor. Here we present the first example of a physical system with this property. Perturbations in such a system render uncertain even the qualitative fate of a given initial state: experiments lose their reproducibility. We suggest that 'riddled' systems of this kind may be by no means uncommon.

The basin of an attractor is said to be riddled if, for any initial condition $\mathbf{r}_0$ in the attractor's basin, the set of $\mathbf{r}$ not in the same

basin as $\mathbf{r}_0$, and within some small distance ε of $\mathbf{r}_0$, has nonzero volume in phase space, no matter how small ε is. Thus a small, random perturbation to $\mathbf{r}_0$ always has a nonzero probability of resulting in a trajectory that does not go to the same attractor as $\mathbf{r}_0$. Therefore, practically speaking, even qualitative determinism (that is, determination just of the eventual attractor, as opposed to the determination of the detailed system state) is in question.

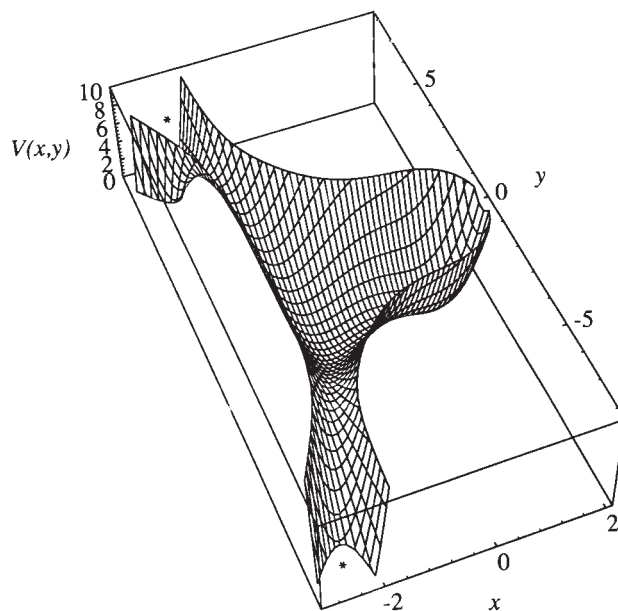


FIG. 1 Shape of the scalar potential. There is an invariant line along $y=0$. A particle finding itself in one of the downturned regions (denoted by *) will be attracted to $y=\pm\infty$.

We consider a unit-mass particle moving in the x - y plane (with two-dimensional space coordinates $\mathbf{r}=(x, y)$)

$$\frac{d^2}{dt^2} \mathbf{r} = -\gamma \frac{d}{dt} \mathbf{r} - \nabla V(\mathbf{r}) + \hat{\mathbf{x}} p \sin \omega t \quad (1a)$$

where the acceleration of the particle is given by the sum of the following forces: (1) a frictional force opposite and proportional to the velocity of the particle, (2) a force given by the gradient of a scalar potential $V(\mathbf{r})$, and (3) a periodic external force in the x -direction. The scalar potential

$$V(x, y) = (1 - x^2)^2 + (x - \bar{x})y^2 \quad (1b)$$

is shown as an energy surface in Fig. 1. In this paper we consider the parameter values $\gamma=0.05$, $p=2.3$, $\omega=3.5$, and $\bar{x}=-1.9$. This continuous-time dynamical system has a five-dimensional phase space: two configuration space coordinates, x and y ; two conjugate velocities v_x and v_y ; and the phase of the forcing term, $\omega t \pmod{2\pi}$. To simplify the system, we study it in the Poincaré surface of section⁴ $\omega t \pmod{2\pi}=0$. This procedure yields a four-dimensional discrete-time mapping, written formally as

$$\xi_{n+1} = F(\xi_n) \quad (2)$$

which takes a phase-space point $\xi_n \equiv (x(t_n), y(t_n), v_x(t_n), v_y(t_n))$ at some time $t=t_n$ (such that $\omega t_n/2\pi=n$, an integer) into a different phase-space point $\xi_{n+1} \equiv (x(t_n+2\pi/\omega), y(t_n+2\pi/\omega), v_x(t_n+2\pi/\omega), v_y(t_n+2\pi/\omega))$ one forcing period later. The dynamical content of equation (2) is identical to that of equation (1) at the stroboscopic sample times.

Note that, owing to the symmetry $V(x, y) \equiv V(x, -y)$, there is an invariant plane $y=v_y=0$ for equation (1): an initial condition started in that plane will remain there for all time. In fact, restricted to the invariant plane, equation (1) is just the forced double-well Duffing equation^{5,6} $(\ddot{x} + \gamma\dot{x} - ax + bx^3 = p \sin \omega t)$, widely studied as a simple model of bistable mechanical and electrical systems. In the invariant plane there is a chaotic attractor, shown in Fig. 2.

The stability of this chaotic set in the whole four-dimensional phase space was analysed in terms of the Lyapunov exponents of equation (2), numerically evaluated along typical orbits in the chaotic set. Lyapunov exponents describe the average relative stability of infinitesimally separated trajectories. Geometrically, the four Lyapunov exponents of equation (2) ($\lambda_1 \geq \lambda_2 \geq \lambda_3 \geq \lambda_4$)

can be interpreted as follows. Given an infinitesimal four-dimensional sphere of radius $\delta\xi$ about an initial condition ξ_0 , the image of the sphere under equation (2) at a very large time $t=2n\pi/\omega$ will be an ellipsoid about ξ_n with principal axes of the order of $\delta\xi \exp(\lambda_i t)$, $i=1, 2, 3, 4$.

Evaluated in the invariant plane, the jacobian matrix $\partial F(\xi)_i/\partial \xi_j$ of the four-dimensional mapping equation (2) has a 2×2 block-diagonal structure, where one of the 2×2 blocks yields information about the relative stability of neighbouring trajectories in the invariant plane (that is, coordinates $(x, 0, v_x, 0)$ and $(x+\delta x, 0, v_x+\delta v_x, 0)$, δ indicating an infinitesimal quantity) and the other block yields information about the relative stability of neighbouring trajectories normal to the plane (that is, coordinates $(x, 0, v_x, 0)$ and $(x, \delta y, v_x, \delta v_y)$). Within the invariant plane, there is one Lyapunov exponent greater than zero ($\lambda_1 > 0$ implies exponentially fast separation of nearby trajectories according to the discussion above; hence the attractor is chaotic), and the other is less than zero. Normal to the invariant plane, both Lyapunov exponents of typical orbits in the chaotic set are negative. This can be shown to imply that a set of initial conditions not in the invariant plane, having nonzero phase-space volume, is attracted to the chaotic set in the invariant plane³. Thus the set is an attractor according to the definition of Milnor⁷. We note, however, that there is a different, often-used definition of attractor, which requires a surrounding open set of initial conditions to be attracted to the candidate set; as we will see, this definition cannot be applied to equation (1). (We also note that the negative normal Lyapunov exponents for typical chaotic orbits in the invariant plane do not rule out a dense set of atypical orbits on the attractor having a positive normal Lyapunov exponent. Such atypical orbits provide a route for initial conditions arbitrarily near the chaotic set to be repelled from its vicinity.)

More interesting is the eventual fate of initial conditions started out (finitely) off the invariant plane. One possibility, established above, is that the initial condition will be attracted to the invariant plane, and will oscillate chaotically within it forever. Another possibility is that the initial condition will eventually find itself in one of the repelling regions of the potential V (indicated by asterisks in Fig. 1) and be pushed off to $\pm\infty$ in y and v_y . Thus there are two 'attractors' for Equation (1): the chaotic attractor in the invariant plane, and the 'attractor' at infinity. As we now demonstrate, the division of initial conditions between these possibilities is not simple.

Figure 3a-d shows the numerically determined fate of initial conditions in a slice $v_x=v_y=0$ of the discrete-time mapping's four dimensional phase space. For each figure, initial conditions on a $1,024 \times 1,024$ grid were integrated to determine their destination. Grid points going to $|y|=\infty$ were plotted as black dots, whereas points going to $y=0$ were left blank. As can be seen from the expanded views (Fig. 3b-d), regions appearing at a given resolution to be attracted to the invariant plane actually contain initial conditions attracted to $y=\pm\infty$. Although the observed fraction of points attracted to the invariant plane increases towards one as one approaches the plane, a blow-up around a point going to $y=0$ will always reveal black dots (when a fine enough grid is used). We note that this behaviour appears to be generic; it is robust to moderate changes in the parameters of equation (1), and presumably to other changes in the potential that do not alter the $y \rightarrow -y$ symmetry.

This combination of (1) a chaotic attractor in an invariant surface in phase space, (2) negative normal Lyapunov exponents for typical trajectories in the attractor, and (3) a positive fraction of initial conditions (in any volume in phase space) attracted to a different attractor, was specified in ref. 3 as the conditions implying a riddled basin of attraction.

The practical consequences of encountering such a system are obviously very serious. Although the underlying equation of motion like equation (1) is strictly speaking deterministic, the riddled geometry of its basin structure, coupled with unavoidable

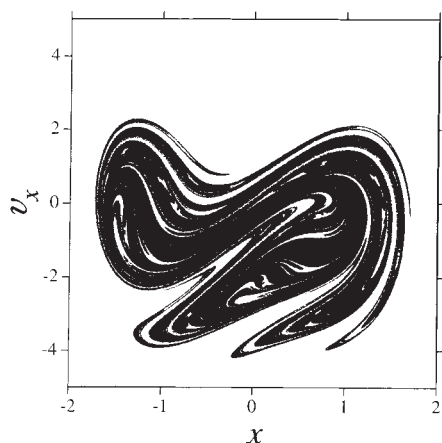


FIG. 2 Chaotic attractor in the invariant subspace $y=v_y=0$. A random initial condition near $x=v_x=0$ was iterated numerically several thousand times to eliminate any initial transient. Then we plotted several hundred thousand iterations. The qualitative nature of the picture is independent of the typical initial condition.

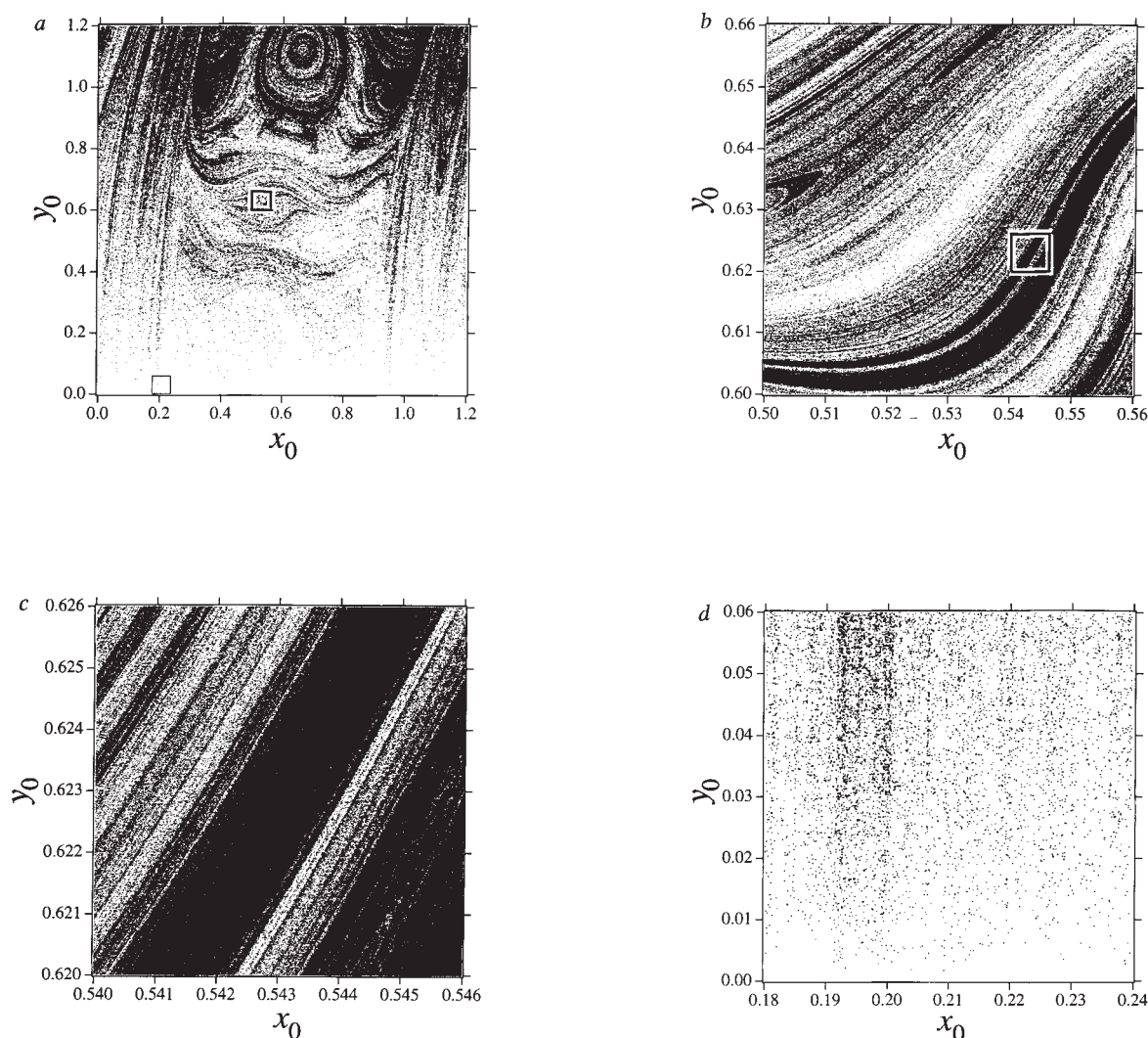


FIG. 3 Destination map for initial conditions in a two-dimensional slice of phase space. Black dots correspond to initial conditions attracted to the invariant plane $y = \pm\infty$. White dots correspond to initial conditions attracted to the invariant plane $y = v_y = 0$. a, $1,024 \times 1,024$ grid. b, $1,024 \times 1,024$ grid giving expanded view of top inset in a. c, $1,024 \times 1,024$ grid giving expanded view of inset in (b). d, $1,024 \times 1,024$ grid giving expanded view of bottom inset in (a) (black dots enlarged for visibility). We chose a single initial condition in each cell of a grid in the phase space, and numerically iterated it forward in time until the trajectory was either

definitely in the repelling region of the potential (taken to be $|y| > 20$, $|v_y| > 100$ and $y \cdot v_y > 0$), or else very close to the invariant plane ($|y| < 10^{-8}$, $|v_y| < 10^{-9}$ and $y \cdot v_y < 0$). The detail, but not the generally riddled character of the picture changes if different initial conditions are chosen at random in each grid cell, or if different computers (with different round-off algorithms and precision) are used to do the calculations. Similarly riddled pictures are obtained for arbitrary slices through the phase space.

perturbations, renders it effectively nondeterministic, and in the worst possible way. Due to measurement errors, simple prediction of the qualitative outcome of even a perfectly modelled experiment would be impossible. Even the reproducibility of apparently identical serial trials (in reality, each starting at imperceptibly differing initial conditions) is in question. From a different viewpoint, unavoidable noise and perturbations during an experiment lead to arbitrarily long transients; a trajectory

perpetually kept off the invariant subspace by noise is eventually attracted to some other attractor. Although we do not expect the conditions specified to be as common as those leading merely to chaos, they are by no means so restrictive that riddled basins can be considered unnatural. (Indeed, we were able to find this behaviour in the first functional form of the potential $V(\mathbf{r})$ that we tried.) Thus, even qualitative reproducibility in simple classical systems cannot be taken for granted. $\square$

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Magnetic bistability in a metal-ion cluster

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MAGNETIC materials of mesoscopic dimensions (a few to many thousands of atoms) may exhibit novel and useful properties such as giant magnetostriction, magnetoresistivity and magnetocaloric effects^{1–4}. Such materials also allow one to study the transition from molecular to bulk-like magnetic behaviour. One approach for preparing mesoscopic magnetic materials is to fragment bulk ferromagnets; a more controllable method is to take a 'bottom-up' approach, using chemistry to grow well defined clusters of metal ions^{5,6}. Lis⁷ has described a twelve-ion manganese cluster in which eight of the Mn ions are in the +3 oxidation state (spin $S=2$) and four are in the +4 state ($S=3/2$). These ions are magnetically coupled to give an $S=10$ ground state⁸, giving rise to unusual magnetic relaxation properties^{8,9}. Here we report that the magnetization of the Mn_{12} cluster is highly anisotropic and that the magnetization relaxation time becomes very long below a temperature of 4 K, giving rise to pronounced hysteresis. This behaviour is not, however, strictly analogous to that of a bulk ferromagnet, in which magnetization hysteresis results from the motion of domain walls. In principle, a bistable magnetic unit of this sort could act as a data storage device.

The structure⁷ of Mn_{12} is sketched in Fig. 1. The cluster has an overall D_{2d} symmetry. The manganese (iii) ions define the external octagon, whereas the manganese (iv) ions correspond to the internal tetrahedron. In the crystal lattice all the molecules have the S_4 symmetry axis parallel to the crystallographic c axis, and the shortest inter-cluster contacts between metal ions are longer than 7 Å. We can consider the ground state as having all the manganese (iii) spins of a cluster up and the manganese (iv) spins down.

The temperature (T) dependence of the single-crystal a.c. magnetic susceptibility (Fig. 2) shows that $\chi'T$ parallel to the tetragonal axis of the cluster reaches a plateau of ~ 143 e.m.u. K mol⁻¹ at 8 K, while at the same temperature the value of $\chi'T$ perpendicular to c is only 10 e.m.u. K mol⁻¹ and the two become equivalent only above 80 K (χ' is the real part of the magnetic

susceptibility χ). The decrease observed below 7 K is due to the onset of the out-of-phase component of the susceptibility, χ'' . High-field magnetization studies confirm that the ground state has $S=10$ and that the magnetic anisotropy is fairly large. In fact, when the field is applied parallel to the c axis the magnetization is saturated below 2 tesla (T), whereas even at 2 K a field of 10 T is required to align the magnetic moments perpendicular to c .

The out-of-phase component of the susceptibility χ'' shows frequency-dependent peaks, with maxima at 5.7 K at 100 Hz and 6.8 K at 500 Hz. This behaviour is indicative of slow relaxation of the magnetization, which is unusual for a paramagnet without an applied field. In fact, similar behaviour is observed for superparamagnets. The relaxation times have been measured down to 2.1 K and the results are shown in Fig. 3. The relaxation times (τ) have been found to follow an exponential law, analogous to that used for superparamagnets, $\tau = \tau_0 \exp(QV/kT)$ (refs 10, 11), where $\tau_0 = 2.1 \times 10^{-7}$ s and $QV/k = 61$ K (k is the Boltzmann constant and V is the particle's volume). This means that at 2 K the relaxation times are of the order of 2 months. The value of τ_0 is about three orders of magnitude larger than usually found in superparamagnets¹¹. Q is the anisotropy constant and has been evaluated from magnetization measurements to be $Q = 2.36 \times 10^{28}$ K m⁻³. Calculating V yields a mean diameter of the particles of 17 Å, which is of the same order of magnitude as the molecular dimensions monitored by the longest metal-metal distance in the cluster, 8.6 Å.

These relaxation effects are consistent with the large anisotropy in the susceptibility, confirmed by the anisotropy measurements. Electron paramagnetic resonance spectra indicated⁸ a large zero-field splitting, with the $M = \pm 10$ levels lying lowest in energy, and the $M = \pm 9$ levels at ~ 10 cm⁻¹. Only the lowest levels are populated at low temperature, and they are responsible for the large observed Ising magnetic anisotropy.

The origin of the large zero-field splitting is associated with the single-ion anisotropy of the Jahn-Teller distorted manganese (iii) ions. In the $S=10$ ground state all the individual $S=2$ spins are aligned parallel to each other, giving rise to a large resultant to the zero-field splitting.

The slow relaxation of the magnetization observed at low temperature is responsible for the large difference between the zero-field (ZFC) and the field-cooled (FC) magnetization shown in Fig. 4a. The two curves become superimposed at ~ 3 K independent of the orientation, but this temperature does not correspond to an anomaly in the relaxation times, suggesting that it is not associated with a magnetic phase transition. This is confirmed by measurements of the specific heat at low temperature, which show no anomaly down to 1.6 K. The levelling of the magnetization below 3 K corresponds to a much smaller value than that expected for a ferromagnet, H/N (where N is the demagnetization factor and H is the magnetic field strength),

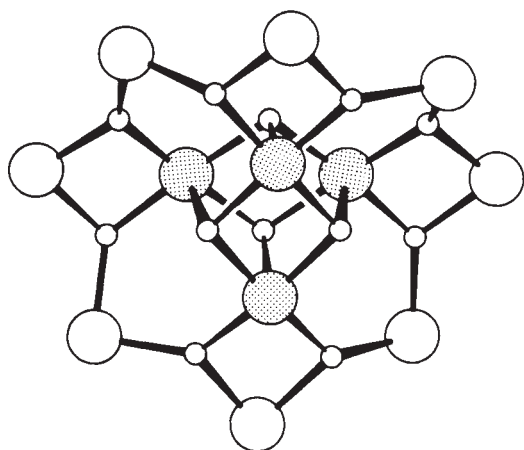


FIG. 1 Schematic view of the core of the Mn_{12} cluster. The largest white circles represent the manganese (iii), $S=2$, ions, the shaded circles represent the manganese (iv), $S=3/2$, and the bridging oxides are represented by small white circles. For clarity we have omitted the 16 acetate bridging ligands and the four water molecules that constitute the cluster. The compound crystallizes in the tetragonal $I4$ space group and the clusters have a crystal-imposed S_4 symmetry axis coincident with the c axis. The shortest distance between centroids of clusters is 12.39 Å.

and is associated with the fact that at that temperature the magnetization is 'frozen' on the timescale of our experiment. Therefore, the behaviour usually associated with bulk magnetism is observed here in a paramagnet.

The compound also exhibits a clear hysteresis loop, as shown in Fig. 4b. Interestingly this hysteresis is substantially different from that of an ordered magnetic material. In this case the applied field is not responsible for the movement of domain walls but for the acceleration of the relaxation process. At 2.2 K the

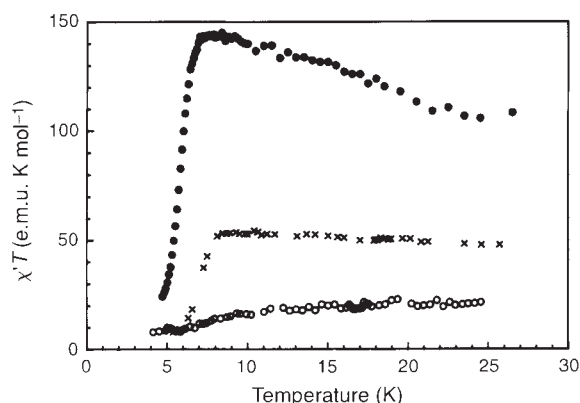


FIG. 2 Temperature dependence of the product of the real part of the magnetic susceptibility (χ') of Mn_{12} with temperature (T) (measured at a frequency of 100 Hz with an a.c. susceptometer) along the c axis (●), perpendicular to it (○), and on a powdered specimen (×). The rapid decrease below 7 K is due to the onset of the out-of-phase component of the susceptibility because of the slow relaxation of the magnetization.

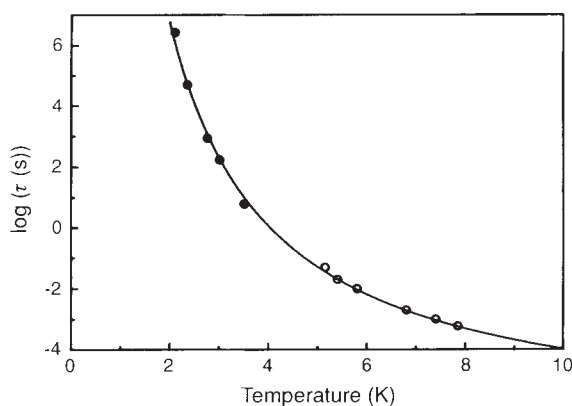


FIG. 3 Temperature dependence of the relaxation time (τ) of the magnetization in Mn_{12} . The solid symbols were obtained by recording the decay of the magnetization whereas the open ones were obtained using the a.c. susceptometer. The solid line represents the best fit with a formula of the type $y = a \exp(b/T)$.

METHODS. Above 5 K the relaxation time at the temperature of the maximum in the out-of-phase susceptibility has been assumed to be equal to ν^{-1} , where ν is frequency of the oscillating field. By plotting χ' and χ'' on a Cole-Cole plot we have found that the points lie on a semicircle, indicating that the relaxation is described by a single characteristic time. The adiabatic susceptibility (χ' when $\nu = \infty$) is different from zero. Below 5 K and down to 2.1 K the relaxation times have been obtained by cooling the sample in a field of 2 T, removing the field and recording the decay of the magnetization with a SQUID magnetometer. The decay follows an exponential law, $M = M_s \exp(-t/\tau)$. At any temperature a small component of the magnetization relaxes fast and is fully reversible, in agreement with the non-zero adiabatic susceptibility observed in the a.c. experiment.

relaxation is sufficiently fast above 1 T for the magnetization to become saturated. When the field is then decreased, the magnetic moments 'freeze' in the aligned position and only at a negative field of ~ 1 T does the magnetic system 'melt' again, and the magnetization reverse. The reversibility field decreases with increasing temperature and the loop becomes narrower (Fig. 4b).

The cluster Mn_{12} shows the typical behaviour of small particles of magnetically ordered materials, such as a blocking temperature which depends on the frequency of the experiment and hysteresis below this temperature. Moreover, it represents the best example of a superparamagnet without particle size distribution and is a model system for testing theories of mesoscopic physics such as quantum tunnelling of magnetization. We feel, however, that the system is not similar to a piece of a three-dimensional magnet; instead, it is better described as a paramagnet, in which the single-ion anisotropy is responsible for the slow relaxation at low temperature. Thus, a superparamagnetic-like behaviour would be a more correct description.

The hysteresis effects described here appear to be of molecular origin (because no evidence of three-dimensional magnetic order was observed in magnetization, susceptibility or specific heat

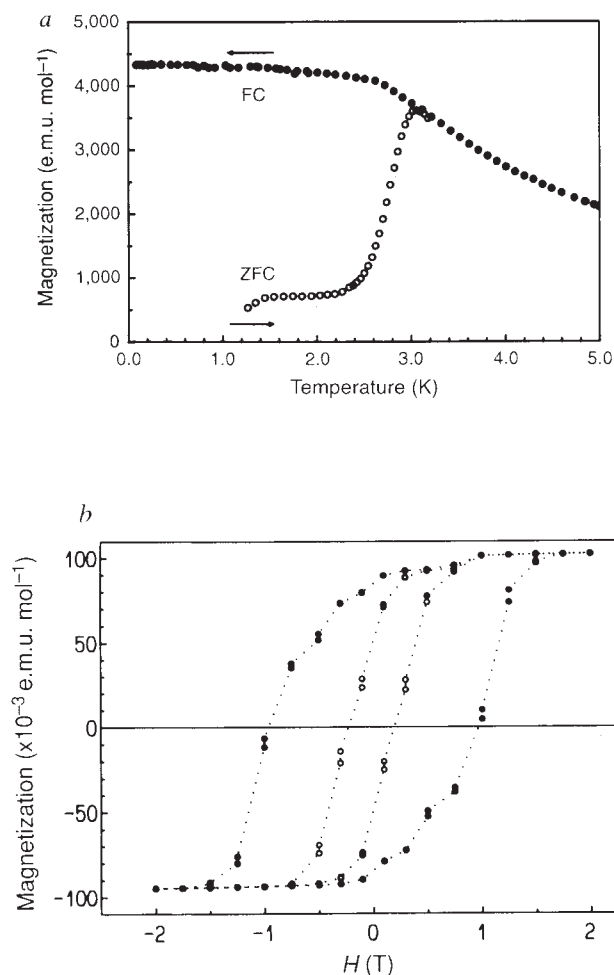


FIG. 4 a, Temperature dependence of the magnetization of Mn_{12} measured parallel to the crystal c axis by cooling the sample in zero field and measuring the magnetization by increasing the temperature in a field of 10 mT (ZFC), and measuring the magnetization by cooling in the same external field (FC). b, Hysteresis loops of Mn_{12} recorded parallel to the c axis with a SQUID magnetometer at 2.2 K (●) and 2.8 K (○). A whole loop was recorded in about 8 h. The dotted lines are only a guide for the eye.

measurements), and this may represent a major breakthrough for molecular materials. This provides the possibility of molecular bistability in which the magnetization becomes 'frozen' in one of two potential wells at low temperature. If a means is found of properly addressing individual clusters it might then be possible to store information at the molecular level—albeit at temperatures no greater than about 4 K in this case. □

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Correlations between climate records from North Atlantic sediments and Greenland ice

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OXYGEN isotope measurements in Greenland ice demonstrate that a series of rapid warm-cold oscillations—called Dansgaard-Oeschger events—punctuated the last glaciation¹. Here we present records of sea surface temperature from North Atlantic sediments spanning the past 90 kyr which contain a series of rapid temperature oscillations closely matching those in the ice-core record, confirming predictions that the ocean must bear the imprint of the Dansgaard-Oeschger events^{2,3}. Moreover, we show that between 20 and 80 kyr ago, the shifts in ocean-atmosphere temperature are bundled into cooling cycles, lasting on average 10 to 15 kyr, with asymmetrical saw-tooth shapes. Each cycle culminated in an enormous discharge of icebergs into the North Atlantic (a 'Heinrich event'^{4,5}), followed by an abrupt shift to a warmer climate. These cycles document a previously unrecognized link between ice sheet behaviour and ocean-atmosphere temperature changes. An important question that remains to be resolved is whether the cycles

are driven by external factors, such as orbital forcing, or by internal ice-sheet dynamics.

Measurements of $\delta^{18}\text{O}$ in the new GRIP ice core from Summit, Greenland provide the first record of air temperatures over the ice cap spanning the last 250 kyr¹. We have correlated the last 90 kyr of that record with a proxy of sea surface temperature in two North Atlantic cores, DSDP site 609 and V23-81 (Fig. 1). The proxy of sea surface temperature is the abundance of *Neogloboquadrina pachyderma* (s.), a planktic foraminifera which lives in waters $<10^\circ\text{C}$ and comprises about 95% of the fauna at summer temperatures $<5^\circ\text{C}$ ^{6,7}. Correlation among the foraminiferal records is constrained by depths of Heinrich events and AMS ^{14}C measurements (Figs 2, 3; and Table 1). Bioturbation of sediment is so low in both cores that we were able to sample at a resolution of 300–500 yr. In addition, by avoiding burrows and other sediment disturbances in V23-81, our new AMS ^{14}C ages have no reversals, a problem that hampered previous radiocarbon dating of that core⁸. We note that ^{14}C measurements in V23-81 and V30-101k revise previous age estimates for Heinrich events 3 and 4 (refs 4, 5) from 28 to 27 kyr BP (before present) and from 41 to 35.5 kyr, respectively (Table 1).

A conspicuous feature common to both the ice and ocean records enabled us to correlate them in spite of their uncertain chronologies. That feature is a bundling of the millennial-scale Dansgaard-Oeschger cycles into longer cooling cycles, each terminated by an abrupt shift from cold to warm temperatures (Fig. 3). We matched the records at the points of abrupt temperature shifts and then 'stretched' the ice-core record linearly until the points were aligned. That alignment is justified by the fact that at the latitudes of our cores (50 – 54°N), shifts in sea surface temperatures must be in phase with air temperature changes above Greenland, especially at the sharp terminations of Dansgaard-Oeschger cycles.

What makes the correlation convincing is that down core, the changes in shapes and internal structures of the longer cycles is nearly the same in all the records (Fig. 3). The stepped pattern of cooling between the Younger Dryas (YD) and ice-core interstadial 1e is closely matched in V23-81, a pattern recognized previously in this core^{8,9} and in the Norwegian Sea¹⁰. Between ice-core interstadials 1e and 2, a 'W'-like pattern with superimposed, short oscillations appears in the ice and sediment,

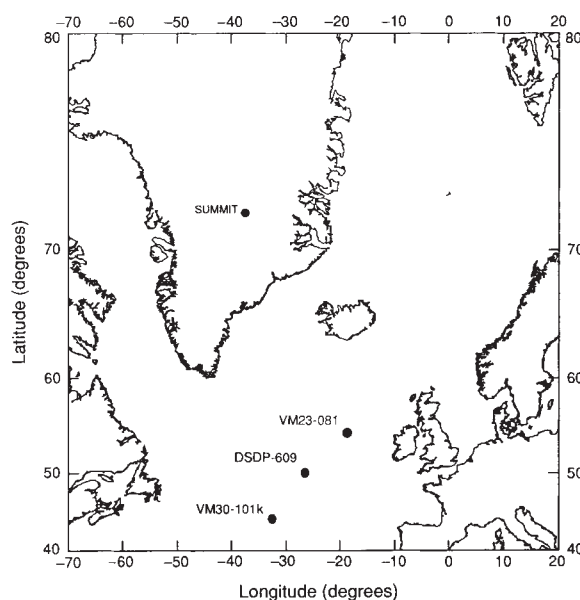


FIG. 1 Locations of the Summit ice core and the deep-sea cores described in this work.

especially in V23-81. Two short interstadials just below the 'W' also appear in V23-81. Between ice-core interstadials 2 and 20 are five asymmetrical cycles, each containing progressively cooler interstadials and culminating in a prominent cold stadial. Much of the structure within these cycles also appears in the foraminiferal records. Finally, the two oscillations on the stage 4/5 boundary and the cooling within marine stage 5b are present in all three records¹¹.

The only direct confirmation of our correlation so far comes from the upper parts of the records where ice-core dating and radiocarbon to calendar age conversions are reasonably well established¹²⁻¹⁴. There, except for the oldest layer, calendar ages of correlated ice and sediment differ by no more than a few hundred years (Table 1, Fig. 3). Two other tests yet to be performed are the depths predicted for marine Ash layers I and II in the ice core (Figs 2, 3).

Our timescale is constrained by ¹⁴C dating for the past ~35,500 years. Below the level of ¹⁴C dating in V23-81 we assigned ages from DSDP site 609 to H5 (50 kyr), to H6 (66 kyr) and to the sharp peak in stage 5b (84.4 kyr) and then interpolated linearly between them. To date the level below 5b in V23-81, we extrapolated the sedimentation rate between H6 and

the sharp peak in 5b. Whereas our timescale is broadly consistent with previous dating of the ice core¹ and with the SPECMAP chronology¹⁵, we emphasize that it is not intended to be an age model for the ice core. Conversion of ¹⁴C ages to calendar ages is uncertain, especially beyond ~20 kyr yr, and sedimentation rates in the marine cores are probably not linear. In fact, the spacing of the ice-core depth intervals should increase steadily down core, and the deviation from that spacing below about 2,200 m (Fig. 3) may be an artefact of changes in sedimentation rates in DSDP site 609.

Our new findings are the strongest evidence so far that Dansgaard-Oeschger cycles are imprinted in the marine sediments of the North Atlantic. The temperature shifts, especially in V23-81, occur on millennium timescales and have asymmetric shapes, sharp boundaries and strong amplitudes (up to a change in temperature of ~5 °C), features which characterize the Dansgaard-Oeschger cycles. A number of the Dansgaard-Oeschger cycles even appear to have direct correlatives in the marine records (Fig. 3). Clearly, for at least 80 kyr the atmosphere and ocean surface were a coupled system, repeatedly undergoing massive reorganizations on timescales of centuries or less. At the abrupt cold-to-warm shifts that terminate

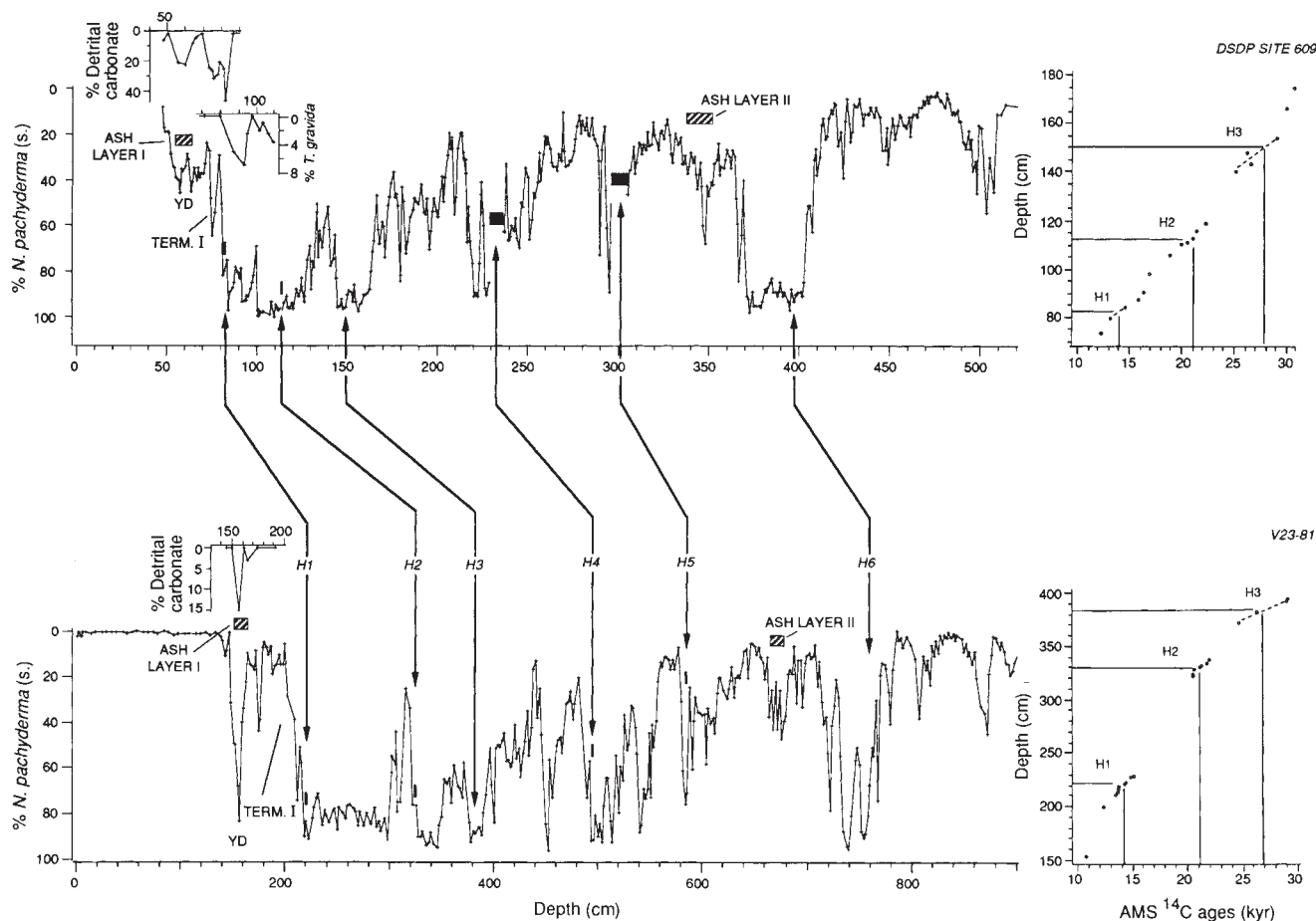


FIG. 2 Abundances of *N. pachyderma* (s.) in two North Atlantic cores, DSDP site 609 (top) and V23-81 (bottom). Samples from DSDP site 609 are from every centimetre; in V23-81, samples are from every 2 cm. Also shown for both sites are new measurements of % detrital carbonate (inset, top and bottom) across the levels of the Younger Dryas (YD) event, demonstrating large discharges of icebergs at that time, presumably from the Laurentide ice sheet. The % of *T. gravida*, a polar diatom (inset, top), indicates a warm-cold oscillation within isotope stage 2 at DSDP site 609. Depths of Ash Layer I and II are based on peak concentrations of the rhyolitic glass that defines those horizons.

Heinrich events, shown as H1 to H6, are brief intervals of increased discharge of icebergs and reduced foraminiferal fluxes that serve as excellent markers for correlation among the marine cores⁴. The AMS ¹⁴C ages to the right of the records are listed in Table 1. In both cores, detrital carbonate-bearing layers within Heinrich deposits are indicated by the black bars (Fig. 2). At DSDP site 609 the detrital carbonate-rich layers at H2, H4 and H5 accumulated nearly instantaneously⁴ and are so thick that they must be removed before constructing the time series. In core V23-81, none of the layers containing detrital carbonate are thick enough to require removal.

Dansgaard-Oeschger cycles (Fig. 2), rates of change in ocean temperatures must have been nearly the same as in the ice core; that is, several degrees within decades^{9,13,16,17}. The large amplitudes and high rates of these temperature shifts are independent of ice volumes, occurring during deglaciation, during the glacial maximum, during the warmer isotope stage 3 and even during the inception of large ice sheets at the stage 4/5 boundary.

Perhaps the most important finding of our study is evidence of an unexpectedly close relation between the ice-core temperature cycles and one of the most prominent features of North Atlantic records, the Heinrich events. Heinrich events occur during times of sea surface cooling, reduced fluxes of foraminifera and brief, exceptionally large discharges of icebergs from the Laurentide ice sheet that left conspicuous layers of detrital carbonate in deep sea sediment^{4,5,18}. Accompanying these events were large decreases in planktic $\delta^{18}\text{O}$ (Fig. 3), evidence of lowered surface salinities probably caused largely by melting of the drifting ice⁴.

Our correlation demonstrates that the iceberg discharges and salinity drops must have occurred during times of particularly cold stadials (Fig. 3). Supporting this is evidence that H1 has the same calendar age, within error, as the younger cold cycle in the 'W' (cycle a, Fig. 3, Table 1). In addition, the Younger Dryas, which unquestionably correlates with a prominent stadial in the ice core (Fig. 3), has features in common with Heinrich events. During the Younger Dryas, North Atlantic sea surface temperatures dropped¹⁹, salinities decreased²⁰, and Laurentide ice advanced through Hudson Strait, depositing detrital carbonate-bearing sediment in the Labrador Sea²¹. We have now found elevated percentages of detrital carbonate in sediments of Younger Dryas age (Fig. 2), indicating that, just as during the Heinrich events, icebergs drifted from the Labrador Sea far into the North Atlantic⁴. We note that the match of Heinrich events to cold stadials confirms our earlier suggestion that Heinrich events formed during periods of extreme atmospheric cooling⁴.

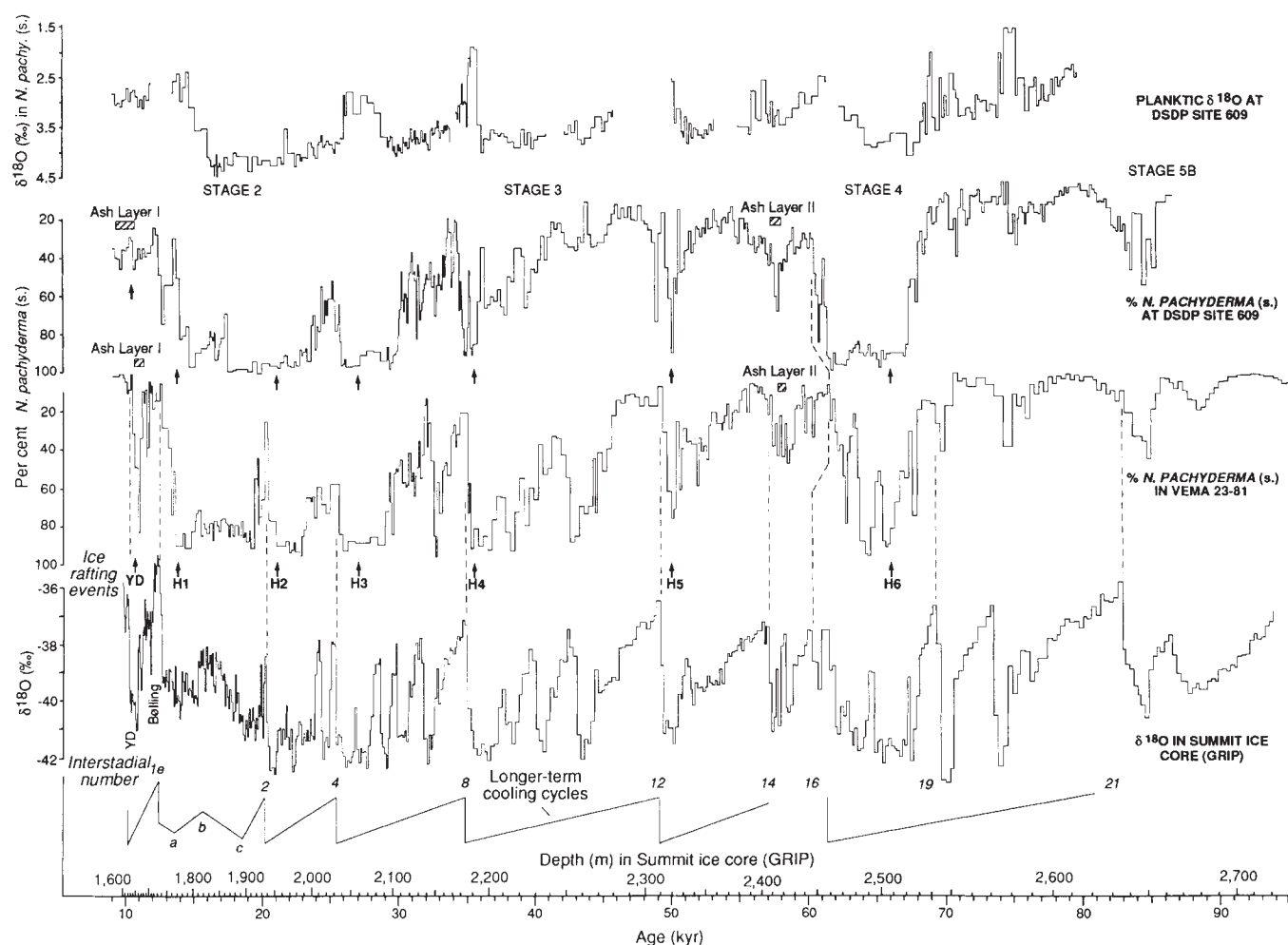


FIG. 3 Our correlation of the foraminiferal records from DSDP site 609 and V23-81 with the new $\delta^{18}\text{O}$ record from GRIP, Summit, Greenland. (Note that the timescale is not an age model for the ice core.) The interstitial numbers in the ice-core record are from the numbering system in ref. 1. Also shown are the longer-term cooling cycles, defined by bundling of millennium scale cycles (Dansgaard-Oeschger cycles) and abrupt shifts to markedly warm interstadials. The dashed lines were used as tie points for matching the ice-core and marine records. The bend in the tie point corresponding to interstadial 16 probably reflects a small decrease in the sedimentation rate at this depth in V23-81 relative to DSDP 609. See text for construction of the timescale. At the top of the figure is the planktic $\delta^{18}\text{O}$ measured in *N. pachyderma* (s.)⁴.

The heavy arrows mark the locations of the IRD peaks within the Heinrich events (H1 to H6) and the peak concentrations of detrital carbonate IRD in the Younger Dryas event. The boxes filled with slanted lines are the levels of Ash Layers I and II in the marine cores; the correlates of these layers in the ice core should occur at depths of ~1,630 m (Ash Layer I) and ~2,426 m (Ash Layer II). It should be noted that the short cooling cycle at about 13,000 ^{14}C yr BP in DSDP site 609, a contemporaneous decrease in the rate of warming in V23-81 and the cold peak containing H1 in V23-81 between 14,000 and 15,000 ^{14}C yr were not recognized previously^{4,5}. These revisions are a consequence of our new foraminiferal measurements in both cores and the improved ^{14}C dating in V23-81.

TABLE 1 Radiocarbon ages for cores DSDP 609, V23-81, and V30-101k

Core	Depth (cm)	Corrected age* (yr)	Error (±yr)	Core	Depth (cm)	Corrected age* (yr)	Error (±yr)
DSDP 609	73–75	12,350*	220	V23-81	154–155	10,900†	140
DSDP 609 (G.b.)	79–81	13,250*	090	V23-81	198–199	12,320†	220
DSDP 609	84–85	14,590*	230	V23-81	210	13,440§	120
DSDP 609	87–88	15,960*	240	V23-81	213	13,600§	120
DSDP 609	90–91	16,360*	150	V23-81	217	13,610§	100
DSDP 609	98–99	16,960*	120	V23-81	219	13,630§	100
DSDP 609	105–107	18,940*	220	V23-81	221	14,150§	110
DSDP 609	110–111	19,970*	330	V23-81	223	14,330§	100
DSDP 609	111–112	20,550*	260	V23-81	227	14,770§	110
DSDP 609	112–113	21,110*	220	V23-81	229	15,040§	110
DSDP 609	115–116	21,370*	220	V23-81	321	20,420§	180
DSDP 609	118–120	22,380*	340	V23-81	323	20,470§	160
DSDP 609 (G.i.)	139–141	25,260*	440	V23-81	327	20,570§	180
DSDP 609	147–149	26,170*	310	V23-81	329	20,990§	170
DSDP 609	153–155	29,170*	660	V23-81	331	21,210§	170
DSDP 609	166–167	30,080*	680	V23-81	333	21,700§	180
DSDP 609 (G.i.)	174–176	30,720*	730	V23-81	337	21,960§	190
V30-101k	56–57	23,570§	210	V23-81	371	24,680§	200
V30-101k	70	33,250§	510	V23-81	381	26,270§	260
V30-101k H4† (80 cm)–	85	36,570§	650	V23-81	391	28,980§	320
				V23-81	393	29,050§	310

Ages of interstadial 1e and the 'W' (levels a, b and c) in Fig. 3

Level	¹⁴ C age (yr) in marine cores	Calendar age (yr) in marine cores	Ice core age¶
1e	12,300	14,300	14,500
a	13,550	16,000	16,400
b	15,400	18,440	18,800
c	18,400	21,970	21,070

* Corrected for assumed 400-yr difference between surface-water carbon and atmospheric carbon; G.b. = analyses on *G. bulloides*; G.i. = analyses on *G. inflata*; all other analyses done on *N. pachyderma* (s).

† H4 in V30-101k is Heinrich layer 4 (this layer was initially identified as H3 in ref. 4; subsequent analyses of that core now demonstrate that it is H4).

‡ ref. 8.

§ AMS ¹⁴C ages from analyses done at ETH-Zurich for this work.

|| ref. 14.

¶ ref. 12.

* ref. 4.

It is also significant that except for H1, the Heinrich events, and the Younger Dryas ice rafting event as well, consistently occur near the ends of the bundled Dansgaard-Oeschger cycles that constrain much of our correlation (Fig. 3). The bundles form a series of saw-tooth shaped cycles, each defined by a succession of progressively cooler interstadials (Fig. 3), probably reflecting a progressive strengthening of the polar cell. Each cycle culminates in a prolonged cold period (stadial) during which a Heinrich event occurs. Following the stadial is a rapid termination-like shift to a prominent warm interstadial marking the beginning of the next cycle (Fig. 3). This complex pattern, known previously only from deglacial records^{8,10}, persists through almost all of the last glaciation, the only exceptions being the intervals through the 'W' and stage 5b.

The series of saw-tooth shaped cooling cycles is clearly a fundamental structure of the atmosphere and sea-surface records, and must bear a close relation to the Heinrich events and the repeated, massive collapses of the Laurentide ice sheets. With the evidence in hand we cannot be certain whether the cooling cycles were caused entirely by internal oscillations of the ice sheet²², or whether they reflect a mode of climate forcing that caused ice sheets to grow, culminating each time in a prolonged, cold stadial, ice-sheet instability and massive calving. If internal oscillations of the ice sheet are sole mechanisms, they must have operated during times such as the Younger Dryas when ice volumes had decreased. We note however, that because H1 comes at the end of a different type of cycle, the 'W', its relation to

climatic and ice-sheet mechanisms may well have been different from that of the others.

The abrupt warmings that followed the Heinrich events and that caused the asymmetry of the cooling cycles, on the other hand, could have been a direct consequence of the ice. Collapse of the ice sheet and landward retreat of ice streams after each event must have reduced the flux of icebergs to the open ocean. The resulting increase in surface salinity could have been large enough to strengthen thermohaline circulation, rapidly bringing heat into the North Atlantic. If so, that further supports our suggestion⁴ that iceberg meltwater strongly influenced the North Atlantic's thermohaline circulation during glaciation.

Whatever their origin, the series of asymmetric cooling cycles and the prominent warmings that terminate them are so conspicuous in the atmospheric and ocean surface records of the North Atlantic that they must be imprinted in other records from the last glaciation. In fact, a series of prominent cycles on timescales of several thousands of years appears within marine stage 3 in summer sea surface temperature estimates from other North Atlantic deep-sea cores^{23,24}, in the Grand Pile pollen record²⁵, and in high-resolution benthic $\delta^{18}\text{O}$ records from core V19-30 in the eastern equatorial Pacific²⁶. Perhaps the sea-level highstands demarked in the flights of the marine terraces of the last glaciation²⁷ are the consequence of massive ice sheet collapses during Heinrich events and the warmings at the ends of the cycles. To test these ideas, however, will require the analysis of records with more precise chronologies and at much higher

resolution than has been attempted to date.

Our new findings still leave unexplained the Dansgaard-Oeschger oscillations in air temperature over Greenland that punctuate the intervals between the Heinrich events. Perhaps, as we suggested earlier³, these reflect the salt-induced ocean circulation oscillations caused by an interaction between the ocean heat-pump and the Scandinavian ice sheets. Although many questions remain unanswered, our correlation of the Heinrich events so prominent in the ice cores with the more frequent Dansgaard-Oeschger events in the ice cores brings us significantly closer to understanding the complex link between ice-sheet dynamics and ocean operation. □

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Sharpness of upper-mantle discontinuities determined from high-frequency reflections

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An understanding of the nature of seismic discontinuities in the Earth's upper mantle is important for understanding mantle processes: in particular, the amplitude and sharpness of these discontinuities are critical for assessing models of upper-mantle phase changes and chemical layering. So far, seismic studies aimed at determining the thickness and lateral variability of upper-mantle discontinuities have yielded equivocal results, particularly for the discontinuity at 410 km depth^{1,2}. Here we present short-period (0.8–2.0 s) recordings of upper-mantle precursors to the seismic phase P'P' (PKPPKP) from two South American earthquakes recorded by the ~700-station short-period array in California. Our results show that the 410- and 660-km discontinuities beneath the Indian Ocean are locally simple and sharp, corresponding to transition zones of 4 km or less. These observations pose problems for mineral physics models^{3–5}, which predict a transitional thickness greater than 6 km for the peridotite to β -spinel phase transition. In contrast to the results of long-period studies^{6,7}, we observe no short-period arrivals from near 520 km depth.

Experimental and thermodynamic studies^{3–5,8} suggest that a peridotite to β -spinel phase transition near 410 km depth occurs over a range of 6–19 km, whereas a δ -spinel to perovskite and magnesio-wüstite phase transition near 660 km depth occurs over a smaller range of 1–10 km. Some argue⁹, on the basis of seismic observations of sharp discontinuities, that the upper mantle is chemically stratified, while others¹⁰ propose a non-equilibrium phase transformation as a possible explanation for the observation of sharp boundaries. Topographic variations of ± 20 km on the 660-km discontinuity¹¹ favour phase transitions. A recent seismic investigation¹² of near-receiver converted phases argues for a transitional 660-km discontinuity (20–30 km in thickness)

and a sharp 410-km discontinuity. These results contradict previous studies of P'P' precursors^{1,13–15} that observed a simple and sharp 660-km discontinuity. A paucity of high-frequency P'P' precursors from near 410 km depth^{1,2,15} has made it difficult to determine the sharpness of this discontinuity. This leads some² to suspect defocusing effects due to topography or lateral variations in transitional thickness. Previous studies^{1,2,15} have been limited in their ability to characterize the lateral variability in the sharpness of the 410-km discontinuity, primarily owing to small array apertures and sparseness of observations.

From our survey of large-magnitude South American earthquakes ($M > 6.2$), all clear P'P' arrivals were accompanied by short-period (0.8–2.0 s) P'P' precursors, implying that the 660-km discontinuity beneath the Indian Ocean is simple and sharp, consistent with previous P'P' observations^{1,13–15}. The dense sampling and large aperture of our array (Fig. 1a), combined with the two closely spaced South American earthquakes leads to unprecedented sampling of the upper mantle, enabling us to show the best evidence to date that the 410-km discontinuity is locally as sharp as 4 km thick, but varies laterally over distances of 250–500 km. This lateral variation may explain the paucity of observations and difficulty in measuring the impedance contrast and sharpness of this discontinuity^{1,2,15}.

Numerous investigations of the transitional upper mantle from 200 to 700 km depth have been made over the past fifty years. The thickness and relative velocity contrast of upper mantle discontinuities are best determined from wide-angle P and S waves^{16–18} recorded in the distance range 15° to 30° , near-source^{19,20} and near-receiver^{1,21,22} reflections and conversions, or from PP, SS and P'P' precursors^{1,2,13–15,23–25}.

P'P' (PKPPKP), which is best observed in the distance range 65° to 75° with a caustic occurring near 71° , has been widely studied because its precursors (typically under-side reflections from mantle discontinuities) are ideal for identifying and measuring the transitional thickness of such discontinuities. P'P' precursors are commonly labelled as P'_dP' where *d* denotes the depth of the reflector. We will use this convention, and will refer to the three main upper-mantle discontinuities by their average globally observed depths, that is, the 410-, 520- and 660-km discontinuities^{7,11}.

Since the early 1970s, the United States Geological Survey (USGS) and the California Institute of Technology have been operating regional seismic arrays in California (Fig. 1a). These

consist of 700 high-gain, short-period (1 Hz), vertical-component seismic stations that are ideal for recording low-amplitude, high-frequency P'P' precursors. The two South American events discussed here represent the best examples of P'P' and its upper mantle precursors recorded by this array since we began looking for P'P' phases in June 1990. The region of the mantle sampled by these earthquakes spans approximately 600–1,000 km beneath the Indian Ocean (Fig. 1b), with each event sampling a portion of the mantle 250 km apart.

Shown in Fig. 2 are the record sections of the P'P' window for two South American earthquakes recorded in California. The Peru–Brazil event was well-recorded at 140 stations (Fig. 2a), whereas the southern Peru earthquake was well-recorded on 231 stations (Fig. 2b). P'P' is observed in the period range 0.7–4.0 s, whereas the precursors are clearly observed between 1.0–2.0 s and weakly observable between 0.8–1.0 s. A dominant period of 1.5 s for the precursor is comparable to a dominant period of 1.2–3.0 s in previous studies^{1,15}. Both profiles (Fig. 2) show clearly a P'₆₆₀P' phase, while P'₄₁₀P' is observed from only one event. From the Peru–Brazil earthquake (Fig. 2a), P'P' (2,275–2,290 s), P'₆₆₀P' (2,140–2,160 s) and P'₄₁₀P' (2,190–2,210 s) are observed from 64° to 71°. The southern Peru earthquake (Fig. 2b) produced P'P' (2,320–2,345 s) and P'₆₆₀P' (2,180–2,210 s) from 68° to 75°. The P'₄₁₀P' phase is most likely to be observed near its caustic, which is at about 72°, but the record section (Fig. 2b) shows no evidence for such a phase.

Estimates of the sharpness of the 410 and 660-km discontinuities can be determined from P'P' observations^{15,26}, but depths to

the reflectors are difficult to ascertain. This is due to imprecise measurement of the arrival time and poorly known upper-mantle and crustal-velocity structure. A change of 10 km in the depth of the 660-km discontinuity produces a travel-time difference of ~2 s for the precursor, which is small given the uncertainties in the velocity structure. Comparing the observed data with theoretical arrival times using the IASP91 reference model²⁷, estimated reflector depths of the precursors are measurable to ±10 km. We find a depth of 650 km beneath the Indian Ocean, which is consistent with longer period estimates of <650 km for the same region²⁸.

Waveform similarity and a dominant period of 1.5 s for the P'₄₁₀P' and P'₆₆₀P' precursors (Fig. 3a) suggest that the 410- and 660-km discontinuities sampled by the Peru–Brazil earthquake are simple and sharp. One would expect to observe relative frequency and waveform differences for either a transitional discontinuity or topography on the discontinuity, neither of which are observed.

No evidence of a sharp seismic discontinuity (thickness <4 km) near 520 km depth is found in the stacked traces (Fig. 3b). The stacked record, aligned on P'₄₁₀P', displays uniform coda levels in the time window appropriate for a precursor from

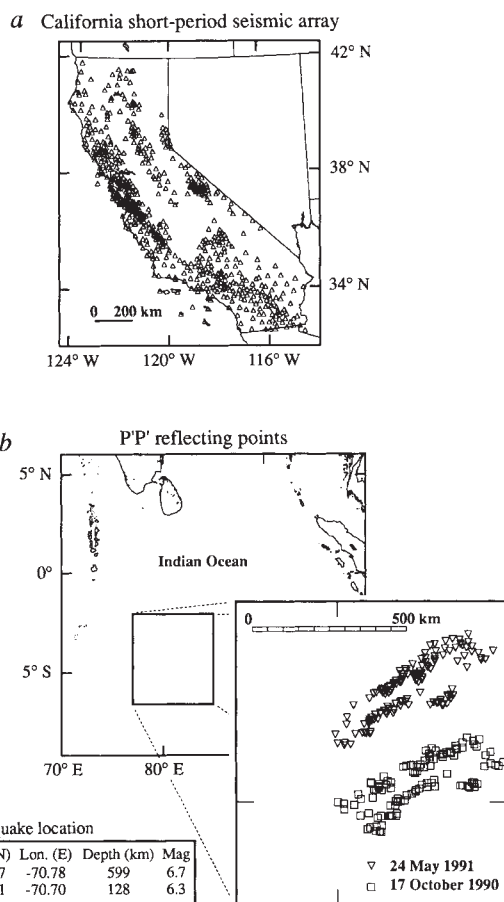


FIG. 1 a, Location of the approximately 700 short-period, vertical-component seismic stations (triangles) operated in California by the US Geological Survey and the California Institute of Technology. b, Location of the mid-point reflections of the P'P' phase from two South American earthquakes recorded by the California short-period seismic array.

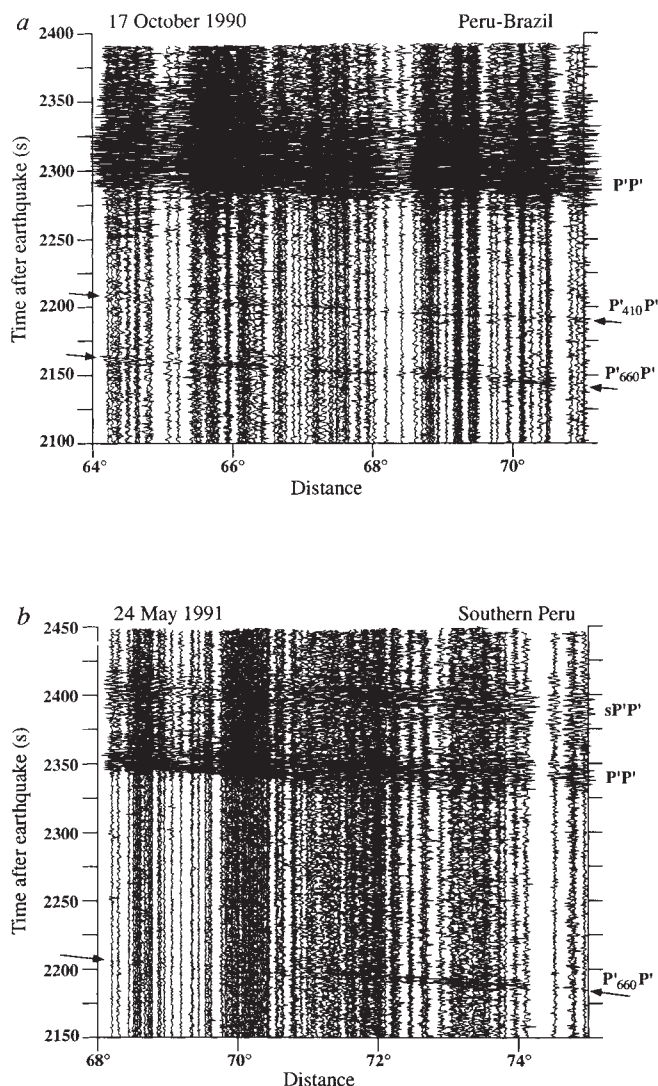


FIG. 2 P'P' record sections for two South American earthquakes recorded by the California seismic array. Each trace is normalized to a constant width, and only traces with a signal-to-noise ratio greater than 5 are shown.

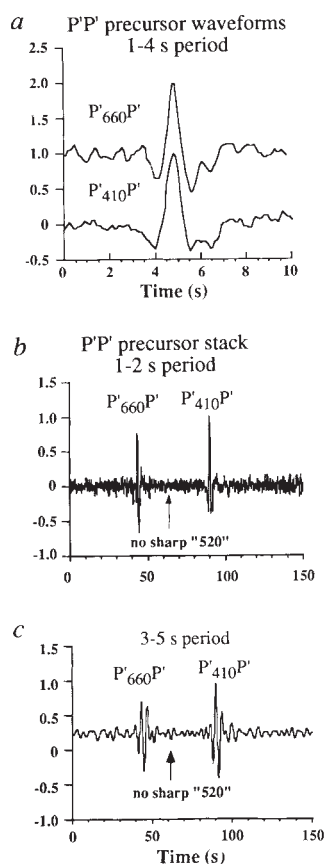


FIG. 3 a, Comparison of the precursor waveforms from stacking the 70 seismograms that recorded both $P'_{410}P'$ and $P'_{660}P'$ phases. Each seismic record was aligned on the appropriate precursor before summing. The similar frequency content and waveform shape for the two precursors argue for simple and sharp velocity discontinuities. The y axes in this figure represent normalized amplitude. b, Stack of upper-mantle precursors showing no high-frequency evidence for a precursor near 520 km depth. The stack was produced by aligning records on the $P'_{410}P'$ phase before summing. c, Low-frequency stack of upper-mantle precursors, demonstrating that the transitional thickness of a 520-km discontinuity must be greater than 10 km.

near 520 km depth. A low-frequency stack (Fig. 3c) also shows no evidence for a 520-km discontinuity, which implies that the transitional thickness of this discontinuity must be greater than 10 km. Lack of support for a sharp discontinuity near 520 km depth is consistent with wide-angle, short-period observations in Australia²⁹ and southern California³⁰.

Several studies of $P'P'$ precursors^{1,2,15} have noted the paucity of observations from near 410 km depth, which suggests that this boundary may be more complex and difficult to quantify in terms of its impedance contrast and transitional thickness. Factors that may explain these observations are lateral variations in the transitional thickness and impedance contrast of the discontinuity, or topographic relief along the boundary². Synthetic seismogram simulations² show that topographical changes (with wavelengths and amplitudes of the order of 300 km and 10 km respectively) can decrease the amplitude of $P'P'$ precursors by a factor of 2–4. Variations in the impedance contrast and transitional thickness of the discontinuity can decrease the amplitude of a precursor by similar or greater amounts^{15,26}. Each is adequate to explain the lack of the $P'_{410}P'$ precursor for the southern Peru earthquake. Alternatively, focusing effects can increase the observed amplitude by a factor of 2–4, making it difficult to estimate the reflection coefficient at the boundary³. But the observed frequency content of the precursor provides excellent constraints on the maximum transitional thickness of the discontinuity.

We use reflectivity synthetic seismograms to determine the maximum transitional thickness consistent with the $P'_{410}P'$ precursor observed in the frequency range 0.5–1.0 Hz. Because it is difficult to determine an appropriate attenuation model beneath the Indian Ocean, relative amplitudes between $P'P'$ and its precursors are difficult to compare. But the synthetic seismograms can be used to explain relative differences between $P'_{410}P'$ and $P'_{660}P'$, as attenuation between the two discontinuities is not appreciable.

Reflectivity synthetic seismograms for three velocity models (Fig. 4) were computed at frequencies of 0.5–1.0 Hz and a distance (δ) of 70.5°, where the amplitudes of the $P'_{410}P'$ and $P'_{660}P'$ are comparable and $P'_{410}P'$ is most likely to be observed. Synthetic seismograms show a 75% decrease in the $P'_{410}P'$ amplitude for a 4-km-thick transition zone and no observable $P'_{410}P'$ for a 6-km-thick transition zone, relative to a first-order

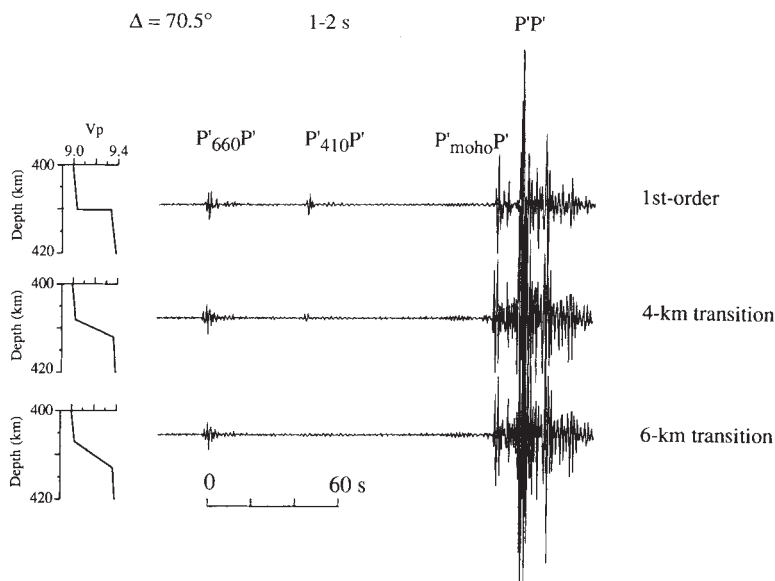


FIG. 4 Reflectivity synthetic seismograms of $P'P'$ and its precursors for different thickness of the 410-km discontinuity and a constant distance (Δ) of 70.5°. For all three cases, a discontinuity at 660 km is modelled as a first-order discontinuity, while the 410-km discontinuity is modelled as 0, 4 and 6-km-thick transition zones. Seismograms were computed using the IASP91 velocity model²⁷ and the PREM density model³¹. Upper mantle low velocity zone densities were modified to be consistent with higher IASP91 velocities. Synthetic seismograms were computed in the frequency band 0.2–1.0 Hz with constant P- and S-wave quality factors of 5,000 and 2,000, respectively.

discontinuity. Synthetic seismograms demonstrate that a maximum transitional thickness of 4 km is necessary to generate the observed precursor. Since the amplitude of the reflection is falling off rapidly in the period range 1–2 s for a 4-km-thick transition, a maximum thickness of 2 km is likely. The results are consistent with theoretical reflection coefficient calculations²⁶ that estimate a 4-km-thick transition for 2 s periods and a thinner transition for shorter periods. Our data are not sufficient to rule out either topographic or transitional variations as the cause of the variability of P'_{410} observations. But high-frequency observations and synthetic seismograms clearly show that the 410-km discontinuity is, at least locally, too sharp for its current phase boundary explanation. □

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Functional topographic mapping of the cortical ribbon in human vision with conventional MRI scanners

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THE human brain has anatomically distinct areas in which processing is laid out in space at the millimetre level with substantial variation across individuals. Activity occurs along a cortical ribbon 1.5–3 mm thick¹ in response to specific stimuli^{2,3}. Here we report the first use of cortical ribbon analysis on humans using non-invasive functional magnetic resonance imaging techniques performed with a conventional 1.5 T MRI scanner. Changes in activation were detected using T2*-weighted, gradient echo imaging sequences. Subjects observed partial field, flashing checkerboard patterns (left–right, top–bottom, half rings, and wedges). Stimuli produced magnetic resonance signal changes in the 1–8% range, varying at the millimetre scale, which showed contralateral vertically reflected patterns of activation in the visual cortex. To compare the spatial topographies across subjects, computer algorithms were used to control for the subject-unique folding of cortex, providing a flattened cortical ribbon identifying four topographically distinct areas.

Changes in cortical metabolism and cerebral perfusion have been recorded non-invasively in humans with positron emission tomography (PET)⁴, and functional magnetic resonance imaging (fMRI)^{5–7}, typically with special purpose high-speed imaging devices and/or on high-field MR devices. We used conventional MRI scanners (1.5 T Signa, 4.7 software release; GE Medical Systems, Milwaukee) with standard scanning software and a surface coil centred over the occipital pole. Six normal subjects underwent fMRI imaging. Functional imaging scans

used T2*-weighted, gradient echo sequences with late echo times (TE, 40 ms) based on previous fMRI studies⁸. A multiple slice scanning sequence (flip angle, 30 degrees; repetition time, TR, 100–150 ms; in-plane resolution 1.88 × 0.94 mm) was used to produce 2–6 slices (each 6–10 mm in thickness) per trial. There were 10 trials per condition. Each trial included 10 s of stimulation, 28–60 s of stimulation plus scanning (depending on the number of slices recorded), and a 5-s inter-trial interval. The magnetic resonance parameters used for functional scans are thought to be sensitive to levels of blood oxygenation⁸. Signal dephasing occurs at a microscopic (subvoxel) level in tissues surrounding the vessels when haemoglobin becomes deoxygenated⁹.

The statistical significance of differences was evaluated using a group *t*-test¹⁰ for each voxel (0.93 × 0.93 mm volume element) of the image. Owing to the use of a surface coil, the signal magnitude decreased with distance from the pole (reaching 50% at 5 cm). Voxels with less than 50% signal were excluded from the analysis. We used several criteria: to identify the extent (in voxels and mm²) of activated regions, an α -level of $P < 0.05$ (one-tailed t (d.f. = 18) > 1.73) was used, and to show the reliability of the effect, criteria at the $P < 0.005$ and $P < 0.0005$ levels are provided. In addition, we statistically defined regions of interest through particle analysis, rejecting voxels that were not part of a contiguous set of at least 16 voxels on sagittals, and 8 on coronals (with either sides or corners touching).

On each trial, the subject was presented with a partial field reversing checkerboard pattern, reversing at a rate of 8 Hz^{5,11}. The subject's task was to monitor for rare targets (average 1 per 10 s) of either a brief (50 ms) flash of a white square, or movement of a fixation cross. Stimuli were presented by rear-projecting computer-generated images produced on an active matrix liquid crystal display projection panel. The subject viewed the stimuli through prism glasses on a screen at the subject's feet. The visual angles of the stimulus were 18 deg horizontal and 11 deg vertical.

Figure 1 shows sagittal activation images, with the locations of significant differences between the two conditions illustrated in warm (violet, red, yellow) and cool (dark blue, blue, cyan) colours relating to the display. Large areas of significant activation occurred along the lingual and cuneate gyri and in the superior occipital cortex. The activation pattern maps illustrate contralateral (Fig. 1a, b) vertically reflected (Fig. 1c) activation

of the visual cortex with the fovea at the posterior pole (Fig. 1d). This localization is consistent with reports produced by other methods¹², but with higher spatial resolution.

To improve localization of the position of activation along the cortical ribbon, and to maximize in-plane resolution, 4–6 images were recorded from four subjects in a plane perpendicular

to the calcarine fissure as determined by structural images taken at the time of the study. The activity peaked at the third slice (12–18 mm from the pole).

Figure 2 shows contralateral vertically reflected activation of the visual cortex. Note that the right cortex activation (all blue–cyan in Fig. 2a) for left–right, splits vertically in the top–bottom condition (Fig. 2b) and splits again with wedges (Fig. 2c). The control condition (Fig. 2d) shows very little activation, indicating that the statistical procedures produce a few false positive activation areas that are small and not over the areas of interest.

Table 1 shows the areas of activation found per subject in the left–right and top–bottom conditions across two slices in the sagittal/axial orientations and six slices in the coronal orientation. The Table reports those regions that exceeded criterion ($t > 1.73$, $P < 0.05$, particle size > 16 for sagittals, > 8 for coronals). The average change was 3.9%, average $t = 3.20$, average peak t per particle 6.41. The peak t provides an estimate of the centre point of activation and is similar to the peak Z metric often used in PET¹³. Each subject's data showed reliable (peak $t > 3.92$, $P < 0.0005$) activation in response to checkerboard stimuli.

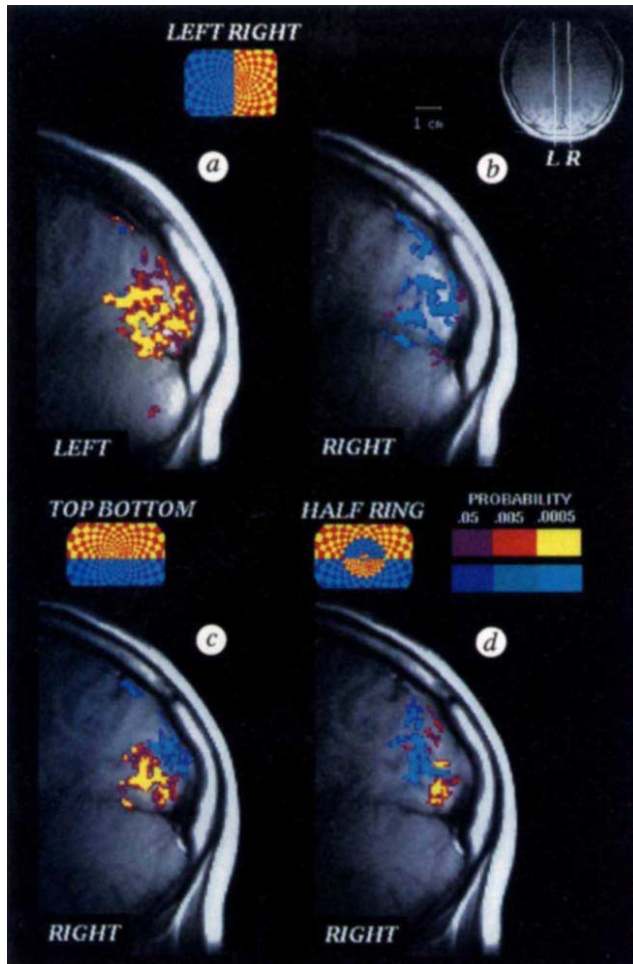


FIG. 1 Sagittal t -test FMRI activation images of subject 1 for 10-mm-thick slices on the mesial wall of the cortex. Grey shows the T1 structural image. Overlaid cool colours (blue–cyan, for positive t -values) and warm colours (violet–yellow, for negative t -values; see colour bar) indicating location of significant t -values (d.f. = 18, $P < 0.05$) of particles of at least 16 adjacent voxels from the differencing conditions: a, left cortex in left–right condition; b, right cortex in left–right condition; c, right cortex in top–bottom condition; d, right cortex in the half-rings condition. Location of the slices is shown in the upper right. The stimulus condition is shown above the cortical images. In the left–right condition (a, b), large areas of significant activation followed the lingual and cuneate gyri. In c, top–bottom activation patterns are shown in the right hemisphere. The area of the right cortex that was activated by the left stimulus in the left–right experiment divided into two parts in c: an upper part in the cuneate gyrus (188 mm², 3.57%, peak $t = 9.12$) and a lower part in the lingual gyrus (294 mm², 3.29%, peak $t = 14.59$). The half-ring stimuli divided lingual activation into three areas: a yellow region at the posterior pole (85 mm², 3.00%, peak $t = 6.27$) representing the upper inside visual ring, a blue region more anterior (166 mm², 2.13%, peak $t = 11.09$) representing the upper middle visual ring, and a small more anterior red region representing the upper outside ring (size 5 mm², 1.16%, peak $t = 3.32$). The upper cuneate gyrus activation from the half ring is divided into two significant regions: the yellow and red region anterior to the pole for the lower middle visual ring (58 mm², 2.06%, peak $t = 5.89$), and a blue region further anterior (58 mm², 1.43%, peak $t = 4.77$) for the outside lower visual ring. The region of activation in the cuneate, near the occipital pole, did not reach criterion.

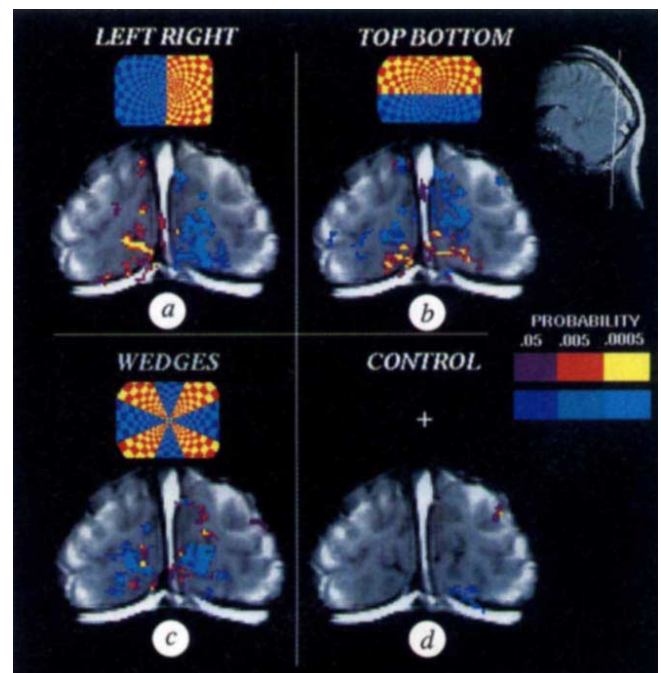


FIG. 2 Coronal t -test FMRI activation images 12–18 mm anterior to the occipital pole on subject 4 for a, left–right; b, top–bottom; c, wedges; and d, fixation-only control. The position of the slice is shown in the upper right, and stimuli are shown above the cortical images. The left–right (a) significant area of activation was 236 mm² on the right side, with a mean change of 5.22%, and on the left was 159 mm², with a mean change of 5.09% (peak $t = 10.73$). There is also a sharp transition of activation from top to bottom stimulus (b). In the left hemisphere, the transition from -1.73 to $+1.73$ t -test values occurred in 2 mm, corresponding to the boundary of the stimulus at the horizontal meridian. The wedge stimulus divided the calcarine fissure region of each hemisphere into multiple positive and negative activation areas. The control fixation-only condition (d), subtracting odd fixation-only trials from even fixation-only trials, shows only small particles reaching the lowest criterion levels ($P < 0.05$), but not the levels exceeded in the stimulus activation conditions ($P < 0.0005$). The lack of large particles provides empirical evidence that the statistical procedure produces few false positives relative to actual activation conditions. In the control condition, there were no significant particles in the left cortex and only one small one (8 mm², peak $t = 2.99$) in the right cortex. The left brain image represents the left side of the subject.

TABLE 1 Areas of activation found per subject in the left-right and top-bottom conditions

Subject	Orientation	Left-right checkerboard								Orientation	Bottom-top checkerboard							
		Left visual field				Right visual field					Lower visual field				Upper visual field			
		Area	t	%	Peak t	Area	t	%	Peak t		Area	t	%	Peak t	Area	t	%	Peak t
1	Sagittal	403	4.55	3.94	11.42	743	4.23	4.06	12.20	Sagittal	321	3.10	3.66	6.81	605	3.77	3.42	10.05
2	Sagittal	213	2.85	3.42	6.29	101	2.89	5.09	6.33	Sagittal	394	2.54	3.59	4.55	241	2.74	2.93	5.43
3	Sagittal	173	2.92	3.25	7.21	102	2.91	3.28	8.04	Sagittal	187	2.79	3.26	4.99	114	2.38	2.83	4.22
4	Axial	484	3.61	3.21	8.38	472	3.41	3.24	7.52	Sagittal	469	3.01	3.52	4.62	99	3.41	4.41	5.51
3	Coronal	729	3.51	4.00	7.50	422	3.38	4.29	6.31	Coronal	380	3.14	3.09	5.41	517	3.15	3.82	6.07
4	Coronal	801	3.71	3.46	8.83	410	3.29	4.61	6.89	Coronal	602	3.71	2.89	7.35	604	3.33	3.45	5.39
5	Coronal	815	3.47	3.70	5.66	688	3.27	3.55	5.04	Coronal	716	2.89	3.33	4.32	1077	2.61	4.24	4.35
6	Coronal	1323	2.98	6.40	4.90	598	3.09	5.93	5.30	Coronal	684	2.89	5.26	4.17	1031	2.76	6.00	4.34

The area is in mm², t is the average t for the region with $P < 0.05$; the % is the per cent change in MRI signal over the region above criterion; the peak t is the average of the peaks of the particles.

To explore further the areas of activation produced by different visual patterns, we performed a cortical ribbon analysis to map out activity across conditions in a given gyrus, as has been done in invasive primate studies³. First, we identified the grey-white border along gyri of interest on T1-weighted images¹⁴. This line was fitted with a third-order Bezier function¹⁵. The activity was measured by taking the average of the per cent change of all voxels that were within 0–2.9 mm of the grey-white border in 0.5 mm units along the line.

The ribbon analysis (Fig. 3) of the calcarine fissure area (region A) differentiates among the four types of stimuli used in the experiments, dividing as the stimulus divides the visual field.

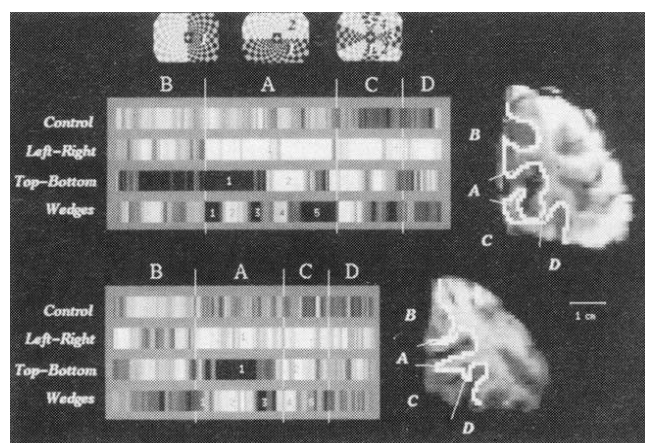


FIG. 3 Unfolding of the cortical ribbon in grey map form. The graph shows the areas of significant activation (black $t < -2$, white $t > 2$). The grey border shows a $t = 0$ value. Numbers label the striping patterns in the calcarine fissure; letters label the region along the ribbon. The cortical slices on the right show the position of the cortical ribbon. Data are from subject 4, slice 3 (12–18 mm anterior from the pole) of the right cortex, with data from 4 conditions along a total length of 136 mm, and for subject 6 (lower panel) for a ribbon of 110 mm in length. The cortical ribbon analysis shows four regions of activation along the line of the upper right cortex. These are: (A) calcarine fissure, (B) medial occipital lobe just superior to the calcarine fissure; (C) medial occipital lobe just inferior to the calcarine fissure, and (D) inferior occipital gyrus/fissure (between lingual and fusiform gyri). Note that the left-right condition dark region of calcarine fissure (A) divides into two in the top-bottom condition and into 5 discernible areas in the wedge condition, matching the expected activity of the visual regions. Regions B and C are active for stimuli in the lower and upper visual field, respectively; region D is differentially active in the left-right experiment but not in the top-bottom experiment. This region receives input from both the upper and lower visual field (based on subtractions from control) which results in no net subtraction in the top-bottom condition. Subject 6 (lower panel) shows a similar pattern, except that the top-bottom transition point is near the lower edge of the calcarine fissure and upper visual field representation continuing beyond the end of the calcarine fissure on the inferior side.

Figure 4 shows the per cent change along the cortical ribbon on the right side, for the control and left-right conditions.

The different activation patterns support the view that there are four visual regions illustrated in Fig. 3. The functional relationships in the activation images are consistent with human post-mortem cortical fibre tracing and PET studies. For example, the areas in human cortex corresponding to functional visual areas have been identified in the primate by comparing callosal connection patterns¹⁶. Area V1 (striate cortex) lies in the calcarine fissure, partially extending out to the mesial wall. This matches our region A. Areas V2 and V3 are divided into two regions, superior and inferior to the calcarine fissure. These receive input from the lower and upper visual fields, respectively, and match our regions B and C. The fourth visual area, potentially corresponding to area V4 in the monkey, is lateral and inferior to the calcarine fissure, along the lingual-fusiform border, receiving input from the upper and lower visual field. This matches our region D. This area receives input from both the upper and lower field (shown as grey in the cortical ribbon, subtracting top-bottom). For subtractions top-fixation or bot-

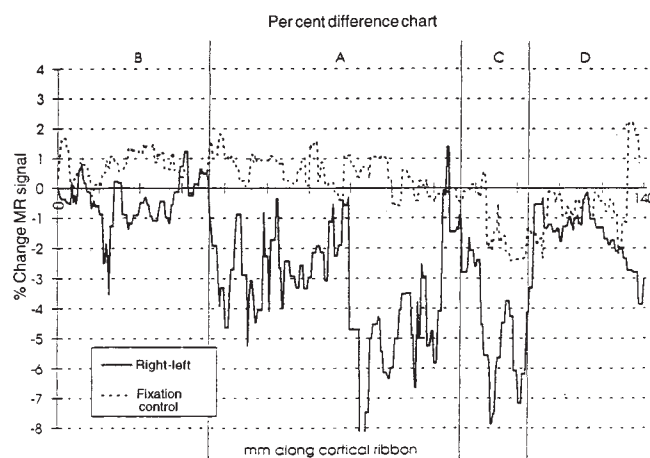


FIG. 4 Per cent change in MR signal along the cortical ribbon for the left-right (solid line) and fixation control (dashed line) conditions for subject 4. Regions along the cortical ribbon are labelled corresponding to the areas in Fig. 3. A negative value indicates left visual field activation; positive value indicates right visual field activation. The per cent change ranges from 1–8% in regions of interest. At the ends of the calcarine fissure, there were positive peaks (positions 31 mm, 1.2%; 93 mm, 1.4% along the ribbon shown) indicating activation from a right stimulus. We interpret these positive peaks in the left-right condition as a point where a portion of the right visual field activates the right cortex potentially through callosal fibres at the visual meridian along the V1/V2 border. Such crossover effects are consistent with primate studies². The high-frequency variation in activation in areas of presumed homogenous activation is similar to the variation seen along the cortical ribbon in 2-deoxyglucose studies³.

tom-fixation, this area has significant activation in both conditions. Our results in this region are consistent with locations identified in PET studies as areas for visual object recognition^{17,18} and for colour processing¹⁹.

Our results demonstrate the application of FMRI methods to obtain high spatial resolution mapping of human cortical activity, allowing the first non-invasive cortical ribbon analysis in humans. Mapping of cortical activity into a flattened cortical ribbon allows identification of common topologies across subjects. Non-invasive millimetre mapping of function over cortex will provide important advances in the understanding of cortical function. □

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Oxygen sensing in airway chemoreceptors

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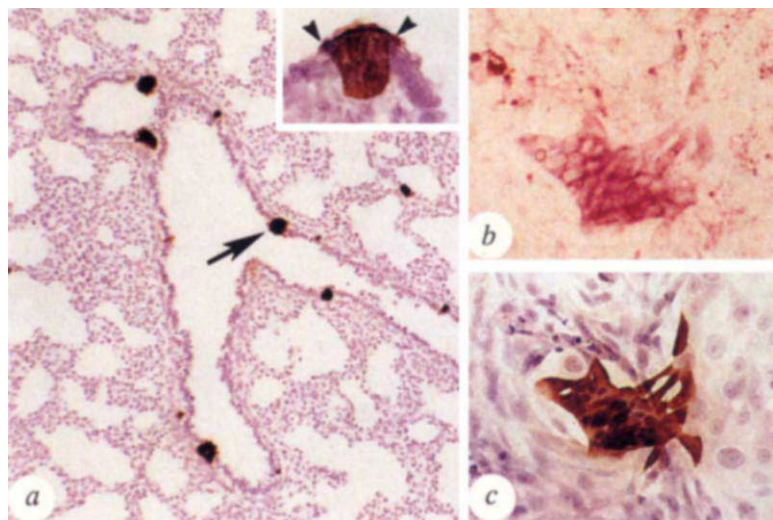
PULMONARY neuroepithelial bodies, composed of innervated clusters of amine- and peptide-containing cells, are widely distributed throughout the airway mucosa of human and animal lungs^{1–3}. Structurally, neuroepithelial bodies resemble chemoreceptors (such as carotid body, taste buds) and are thought to function as hypoxia-sensitive airway sensors⁴. Evidence for this is indirect, however, and the mechanism of oxygen sensing by these cells is unknown. Here we culture neuroepithelial bodies isolated from rabbit fetal lungs and identify voltage-activated potassium, calcium and sodium currents using the whole-cell patch clamp technique. Upon exposure to hypoxia there is a reversible reduction (25–30%) in the outward potassium current, with no change in inward currents. In addition, we demonstrate the expression of an oxygen-binding protein (*b*-cytochrome, NADPH oxidase) on the plasma mem-

brane of these cells. The identification of an oxygen-sensing mechanism (namely the presence of an O₂-sensitive potassium channel coupled to an O₂ sensor protein⁵) in the cells of pulmonary neuroepithelial bodies indicates that they are transducers of the hypoxia stimulus and hence may function as airway chemoreceptors in the regulation of respiration.

Primary cultures were prepared of neuroepithelial body (NEB) cells from lungs of late-gestation (26-day) fetal rabbits (Fig. 1a) as described⁶, with some modifications. To identify NEB in culture and facilitate patch-clamp studies, we developed a technique to visualize NEB cells selectively in the living state. We found that in cultures incubated with the vital dye neutral red (0.02 mg ml⁻¹ for 30–40 min at 37 °C)⁷, NEB cells stained selectively while all other lung cells remained unstained (Fig. 1b). We verified that neutral-red-stained cells were NEB cells using immunostaining with a specific antibody against serotonin (Fig. 1c), a marker for NEB². Electrophysiological recordings were made using whole-cell patch clamp⁸, the cell culture being held in a small chamber where it was exposed to either a bathing solution equilibrated with normal air (*p*O₂, 150 mm Hg) or 100% N₂ (*p*O₂, 25–30 mm Hg). In all cases, bathing solutions were perfused under gravity at a flow rate of 5 ml min⁻¹.

Investigation of the passive membrane properties of NEB cells with whole-cell voltage clamp indicated input resistances of 2.1 ± 0.1 GΩ and capacitance values of 6.1 ± 0.3 pF (mean ± s.e.m.; *n* = 58). Under voltage clamp, depolarizing voltage steps from a holding potential of -60 mV activated both a fast transient inward and a prolonged outward current in NEB cells (Fig.

FIG. 1 a, Serotonin (5-hydroxytryptamine, 5-HT)-immunoreactive NEB in rabbit fetal lung (26-d gestation) located within airway mucosa, particularly near bifurcations (arrow) (× 99). Insert: At higher magnification, tall columnar NEB cells are extending to the airway lumen (arrowheads) (× 231). Formalin-fixed paraffin-embedded sections were immunostained with monoclonal anti-5-HT antibody¹⁹. b, Vital staining of NEB in culture using neutral red⁷. Positive reaction (red cytoplasm with nuclei unstained) is seen only in NEB cells (× 198). To preserve NEB clusters, airway epithelium was dissociated from microdissected bronchial trees (free from alveolar tissue) using 0.2% collagenase (type 1V; Sigma). Epithelial fragments were plated on collagen-coated coverslips and maintained at 37 °C in 5% CO₂ for 3–7 d in MEM or F12 medium (Gibco) with supplements⁶. c, Same NEB as in b, but immunostained for 5-HT (× 198).



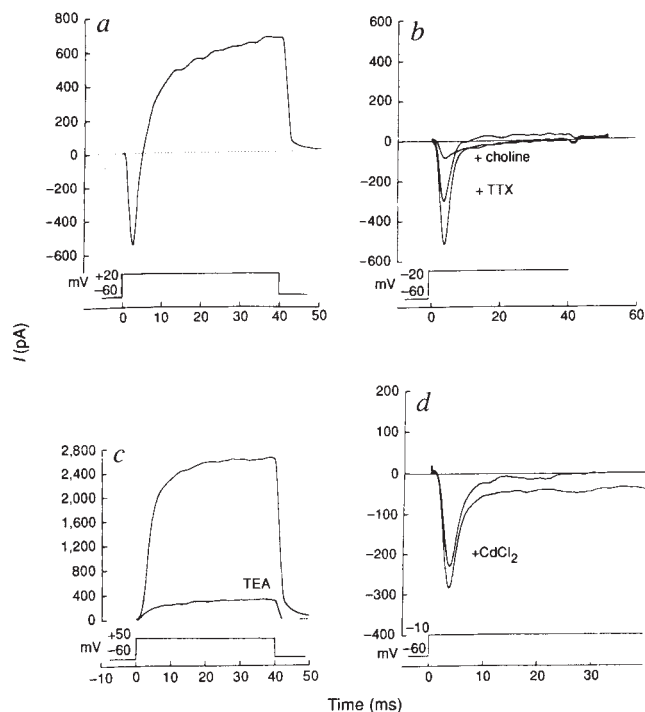


FIG. 2 Whole-cell currents in cultured NEB cells recorded with an axopatch 1B amplifier (Axon Instruments) and a probe equipped with a 1-G Ω headstage feedback resistor. The extracellular solution contained (in mM): (140) NaCl, (1.5) CaCl₂, (3) KCl, (1) MgCl₂, (5) glucose and (10) HEPES, and the intracellular pipette solution (except in d) contained (in mM): (140) KCl, (1) CaCl₂, (10) EGTA and (10) HEPES. Addition of 4 mM Mg-ATP to the pipette solution had no effect on the results. The holding potential was -60 mV with a pulse duration of 40 ms. **a**, Inward and outward currents from a NEB cell recorded in response to voltage step from holding potential to +20 mV. **b**, Inward currents were activated by voltage steps from holding potential to -20 mV. The transient inward Na⁺ current was reduced (82%) by choline (140 mM). A component of this current was blocked (37%) by 2 μ M TTX. **c**, Outward K⁺ current, recorded during a voltage step from holding potential to +50 mV, was reduced (88%) by TEA (12.5 mM). **d**, Combined Na⁺ and Ca²⁺ currents after blockade of outward K⁺ currents (in mM): (105) CsCl, (25) tetraethylammonium chloride, (10) NaCl, (1) CaCl₂, (11) EGTA, (10) HEPES. The slow sustained Ca²⁺ component of the inward current was blocked with Cd²⁺. Voltage traces were plotted after leak subtraction using PCLAMP software.

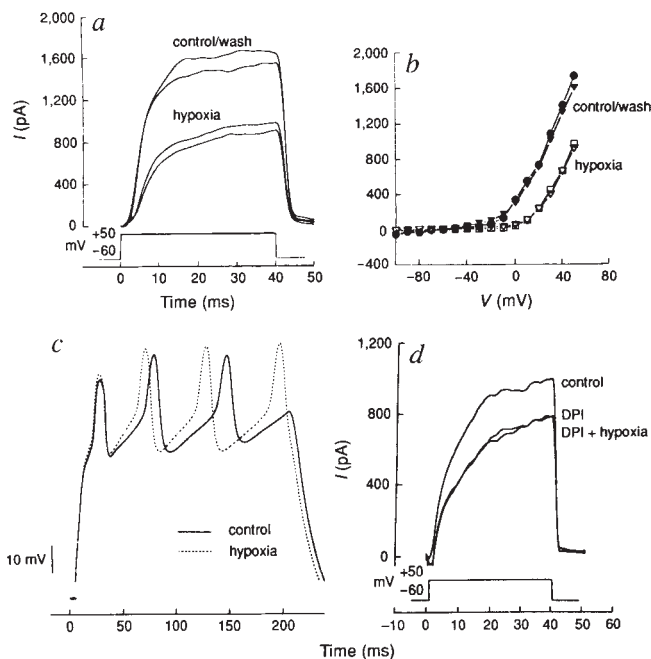
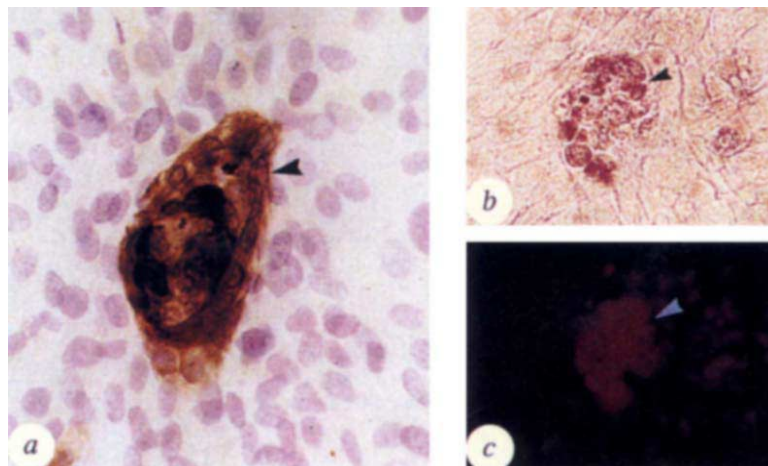


FIG. 3 Effect of hypoxia on outward potassium current in NEB cells. Hypoxia was achieved by bubbling pure N₂ into the bathing medium. **a**, K⁺ currents recorded by voltage steps to +50 mV from a holding potential of -60 mV. The same cell was repeatedly cycled between normoxic (pO₂: 150 mm Hg; control/wash) and hypoxic stimulus (pO₂: 25–30 mm Hg). About 43% reduction of K⁺ current was observed when the cell was first exposed to hypoxia; following recovery in normoxia (wash), a second exposure to hypoxia produced a similar reduction in K⁺ current. **b**, Current-voltage relationship of cell in **a**. **c**, Current clamp recordings from a NEB cell. Repetitive firing was triggered by a brief (5-ms) suprathreshold depolarizing stimulus applied during the period indicated by the horizontal bar. After termination of the stimulus, the spontaneous firing frequency and slope of the depolarizing pacemaker potentials were increased with exposure to hypoxia (dashed line) compared with normoxic control (full line). **d**, Effect of DPI on outward K⁺ current recorded from voltage step of -60 to +50 mV. When NEB were exposed to DPI (0.4–4 μ M), there was a 26% reduction in K⁺ current amplitude which was not altered by further exposure to DPI and hypoxia.

FIG. 4 **a**, Positive immunostaining with specific antibody against the p91 polypeptide of NADPH oxidase appeared to be distributed along the NEB cell membrane (arrow), with adjacent cells negative ($\times 231$). Cultures were fixed in 100% ethanol and immunostained according to the PAP method²⁷. Previously characterized primary antibody¹⁰ (affinity-purified against the 91K heavy chain of b-cytochrome (NADPH oxidase) was used in 1:200 dilution, with overnight incubation at 4°C. **b**, Neutral-red-positive NEB cells in culture before exposure to dihydrorhodamine 123 (Molecular Probes) ($\times 198$). **c**, Same NEB (arrowhead) as in **b**, showing positive rhodamine fluorescence (arrowhead) after 20 min incubation in dihydrorhodamine¹² ($\times 198$).



2a). The inward current was activated at depolarizing voltages between -20 and -30 mV and its peak was variable, ranging from 41 to $1,160$ pA with a mean $\pm$ s.e.m. of 282 ± 34 pA ($n = 51$). The majority of the inward current was carried by Na^+ ions as it was reduced ($72 \pm 7\%$; mean $\pm$ s.e.m.; $n = 5$) when external Na^+ was replaced by the impermeant cation choline (Fig. 2b). In addition, a component of this transient inward current ($43 \pm 5\%$; mean $\pm$ s.e.m.; $n = 12$) was blocked with $2 \mu\text{M}$ tetrodotoxin (TTX), a blocker of voltage-gated Na^+ channels (Fig. 2b). The maximum amplitude of the outward current for a voltage step from -60 mV to $+50$ mV ranged from 394 to $4,260$ pA with a mean $\pm$ s.e.m. of $1,512 \pm 134$ pA ($n = 55$). The outward current was activated between -20 mV and -30 mV and did not inactivate at least over the first 40 ms (Fig. 2a). This current was carried by K^+ ions because it was reduced ($92 \pm 2\%$, mean $\pm$ s.e.m.; $n = 5$) by the K^+ channel blocker tetraethylammonium (TEA, 12.5 – 25 mM) (Fig. 2c) and completely eliminated when caesium and TEA were substituted for K^+ in the pipette solution (Fig. 2d). These last conditions revealed a prolonged inward calcium current which was eliminated when external Ca^{2+} was replaced by cadmium (Fig. 2d).

To test whether NEB cells were sensitive to hypoxia, we first recorded whole cell currents using a normoxic perfusate and then switched to a hypoxic perfusate. Hypoxia reversibly suppressed the outward K^+ current by $26 \pm 4\%$ (mean $\pm$ s.e.m.; $n = 12$) (Fig. 3a). In three cases the same cell was repeatedly cycled (2 – $3 \times$) between normoxic and hypoxic stimuli and in each case a similar suppression was observed. An example is shown in Fig. 3a and b, in which the same cell was cycled between two hypoxic episodes. These findings are supported by current clamp recordings showing an increase in firing frequency and slope of the depolarizing pacemaker potential in NEB cells ($n = 3$) exposed to a hypoxic stimulus (Fig. 3c).

A potential candidate for an oxygen sensor protein⁹ is the haem-linked NADPH oxidase, similar to the one originally identified in neutrophils¹⁰. Using an antibody that recognizes the p91 polypeptide of the oxidase¹¹, we identify the specific localization of this protein in NEB (Fig. 4a) by the immunoperoxidase method. The immunoreactivity appeared to be localized to the plasma membrane of NEB cells. Other epithelial and mesenchymal components within these cultures did not stain for this protein. The uptake and conversion of non-fluorescent dihydrorhodamine to fluorescent rhodamine by NEB cells (Fig. 4b, c) provided histochemical evidence consistent with H_2O_2 production (that is, a functioning NADPH oxidase) as previously shown for the carotid body (CB) type-1 cells¹². A specific inhibitor of NADPH oxidase, diphenylene iodonium (DPI), transiently increases the spontaneous discharge in isolated perfused CB preparations and blocks the hypoxia-induced increase in neural discharge¹². We have now found that under normoxic conditions, DPI causes a reduction in the K^+ current in NEB cells, similar in magnitude to that seen with hypoxia; further, after treatment with DPI, NEB cells no longer respond to hypoxic stimulus (Fig. 3d).

Our observations that late-gestational rabbit NEB express voltage-dependent K^+ , Na^+ and Ca^{2+} currents indicate that these cells have typical neuroendocrine properties. The additional findings that a component of the K^+ current is sensitive to hypoxia as well as our immunocytochemical demonstration that NEB cells crossreact with an antibody against the O_2 -binding protein NADPH oxidase, suggest that these cells might act as transducers of the airway hypoxic stimulus. Similar O_2 -sensitive K^+ currents have been described in CB type-1 cells in rabbits¹³ and rats¹⁴. Like CB type-1 cells, NEB cells show an increase in firing frequency and in the slope of the depolarizing potential with hypoxic exposure¹⁵. The presence of pO_2 -sensitive K^+ channels appears to be a unique feature of O_2 -sensing cells, because hypoxia-induced changes in K^+ current have not been observed in small intensely fluorescent cells (SIF cells), which are morphologically and developmentally related to CB type-1

cells¹⁴, or in petrosal ganglion cells which innervate CB type-1 cells¹⁶. It has been suggested that the oxygen-binding protein is membrane-bound, as in neutrophils¹⁰, and coupled directly to the O_2 -sensitive K^+ channels in CB type-1 cells, because low pO_2 decreases the open probability of K^+ channels in excised membrane patches¹⁷. Our demonstration that the oxidase inhibitor DPI affects the function of K^+ channels is further evidence for a direct link between O_2 -sensitive K^+ currents and the O_2 sensor protein.

Our results support a chemoreceptive function for NEB which operates through K^+ channels to activate secretion of biologically active amines and peptides. As NEB release amine upon hypoxic stimulation *in vivo*⁴ and *in vitro*¹⁸, we propose that the closing of K^+ channels by hypoxia increases spike frequency or initiates the depolarization of the NEB cell membrane, which in turn could open voltage-sensitive Ca^{2+} channels and lead to increased Ca^{2+} influx and neurotransmitter release.

The potential role of NEB as chemoreceptors in the lung and as contributors to the overall homeostasis remains uncertain. The prominence of NEB during the perinatal period^{3,19} indicates that they may be important during the transition from fetal to neonatal life: for example, as airway chemoreceptors, NEB could complement conventional chemoreceptors such as CB and help respiration, perhaps when normal breathing starts at birth and/or during resetting of CB chemoreceptors²⁰. In the neonate, dysfunction of NEB may be important in apnoea of prematurity and also in sudden infant death syndrome, in which hyperplasia of NEB has been reported²¹. In the adult, small cell lung carcinomas express neuroendocrine features similar to normal NEB cells, including amine and peptide neuromodulators^{22,23}; indeed, in the nitrosamine-induced hamster model of small cell lung carcinoma, neoplastic transformation is oxygen-dependent²⁴.

We have shown that NEB cells are transducers of the hypoxic stimulus and hence fulfil the basic requirement of an airway hypoxia-sensitive chemoreceptor. Further investigation should define the function of NEB during neonatal adaptation and in the disorders associated with it, and may uncover new homeostatic mechanisms as NEB have been found universally in gas-exchanging organs²⁵ and to be conserved during phylogeny²⁶. □

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Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50^{csk}

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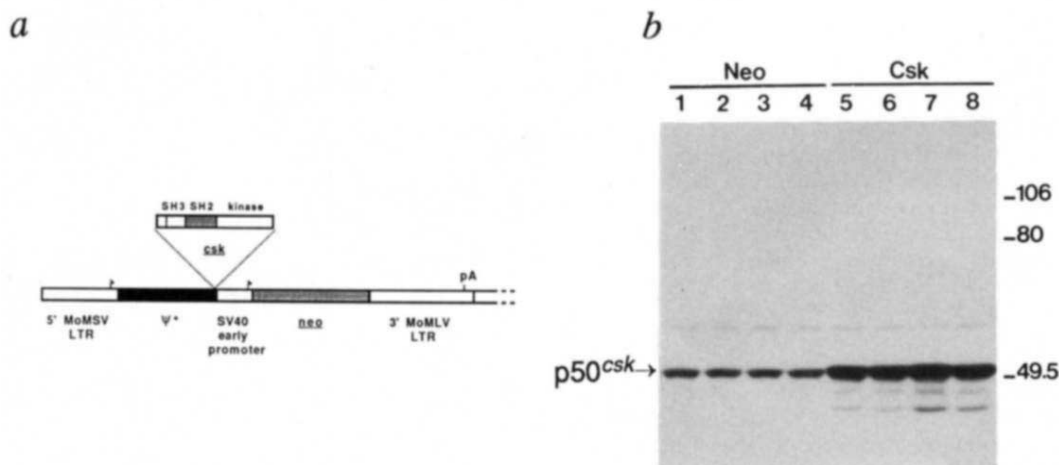
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TYROSINE protein phosphorylation is necessary for antigen receptor-mediated activation of T lymphocytes¹. This signal is generated at least in part by the Src-related tyrosine protein kinases p56^{lck} and p59^{lyn} (refs 2, 3). The activity of these two enzymes is repressed by phosphorylation of a conserved carboxy-terminal tyrosine residue^{2,3}. Recent studies suggest that this inhibitory phosphorylation may be caused by p50^{csk} (for C-terminal Src kinase), a tyrosine protein kinase which accumulates most abundantly in thymus and spleen^{4,8}. To investigate the function of Csk in T lymphocytes and characterize the processes regulating T-cell receptor (TCR) signalling, we examined the effects of overexpression of Csk on the physiology of an antigen-specific mouse T-cell line. We report here that p50^{csk} negatively regulates TCR-induced tyrosine protein phosphorylation and lymphokine production. This provides evidence for the involvement of Csk in the regulation of T-cell activation.

Using retrovirus-mediated gene transfer (Fig. 1a), p50^{csk} was overexpressed in the beef insulin-specific mouse T-cell hybridoma BI-141 (ref. 9). G418-resistant monoclonal cell lines were screened by immunoblotting of total cell lysates with an anti-Csk serum. Several independent cell lines (Fig. 1b, lanes 5–8) containing amounts of Csk 2.5–3-fold higher than those of control neomycin-resistant cell lines (Neo; Fig. 1b, lanes 1–4) were identified. All cells expressed unaltered levels of TCR, CD3ε, ζ, p56^{lck}, p59^{lyn}, CD45 and Thy 1 (data not shown). To examine the role of p50^{csk} in TCR signalling, the effect of p50^{csk} overexpression on TCR-induced tyrosine protein phosphorylation was evaluated. Cells were stimulated with anti-TCR monoclonal antibody F23.1, and the abundance of phosphotyrosine-containing proteins monitored by anti-phosphotyrosine immunoblotting (Fig. 2a). Overexpression of Csk resulted in global inhibition of antigen receptor-induced tyrosine protein phosphorylation (Fig. 2a, lanes 5–12). This phenomenon was consistently observed in seven randomly selected Csk-overexpressing cell lines, and its extent tended to be proportional to the level of Csk expression (data not shown). In contrast, and consistent with our former findings^{10–12}, introduction of activated p56^{lck} enhanced TCR-induced tyrosine protein phosphorylation (Fig. 2a, lanes 13 and 14).

The impact of increased p50^{csk} expression on antigen receptor-induced interleukin-2 (IL-2) production was also tested (Fig. 2b). Cells were stimulated with varying concentrations of anti-TCR antibody F23.1 coated on plastic, and the released IL-2 measured in a standard IL-2 bioassay¹⁰. In five independent experiments, this assay showed that cell lines overexpressing Csk had decreased TCR-induced lymphokine responses compared with control clones (Fig. 2b; and data not shown). But such an effect was partial, as IL-2 release could be recovered by progressively increasing the concentration of anti-TCR antibodies. This was more evident for cell lines having lesser degrees of Csk overexpression.

FIG. 1 Expression of wild-type p50^{csk} in BI-141 T-cells. a, Retroviral construct (pLXSN-csk). The rat csk cDNA was inserted in the polylinker of the retroviral expression vector pLXSN. The construct contains a 5' Moloney murine sarcoma virus (MoMSV) long terminal repeat (LTR), and a 3' Moloney murine leukaemia virus (MoMLV) LTR with its polyadenylation (pA) signal. The neomycin phosphotransferase gene neo (Tn5) is located downstream of the simian virus (SV) 40 early promoter. Expression of this gene allows growth in medium containing the aminoglycoside G418. The position of the ψ¹ packaging sequences is shown. Transcriptional orientations are highlighted by arrows. b, Anti-Csk immunoblot. Levels of p50^{csk} in multiple independent BI-141 T-cell derivatives infected with retroviruses encoding the neomycin phosphotransferase alone (Neo; lanes 1–4), or in combination with wild-type p50^{csk} (Csk wt; lanes 5–8) were measured by anti-Csk immunoblotting. Lanes 1, Neo.2; 2, Neo.3; 3, Neo.4; 4, Neo.5; 5, Csk wt.1; 6, Csk wt.5; 7, Csk wt.28; and 8, Csk wt.54. The relative levels of Csk expression were assessed and compared with the average of Neo cells, using a phospho-imager (BAS 2000; Fuji): wt.1, 2.5-fold; wt.5, 2.7-fold; wt.28, 3.2-fold; and wt.54, 3.1-fold. Exposure, 8 h.



METHODS. The HindIII–HindIII fragment of the rat csk cDNA⁴ was cloned in the appropriate orientation into the HpaI site of pLXSN¹⁶ (from D. Miller, Fred Hutchinson Cancer Center, Seattle, Washington, USA). Transfection in ψ-2 packaging cells and retroviral infection of BI-141 T cells was as described elsewhere^{10,17}. Cells expressing the neomycin phosphotransferase alone were prepared as outlined previously¹⁰. Levels of Csk expression were measured by anti-Csk immunoblotting of total cell lysates¹⁰, using a polyclonal rabbit anti-Csk serum generated against a TrpE fusion protein containing residues 182–450 of rat p50^{csk} (our unpublished data).

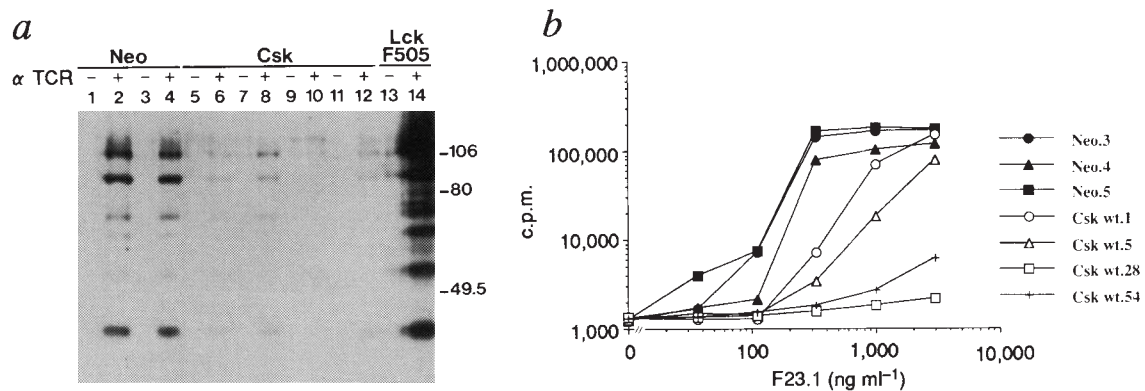


FIG. 2 Effects of p50^{csk} overexpression on antigen receptor-mediated responses. **a**, TCR-induced tyrosine protein phosphorylation. The abundance of phosphotyrosine-containing proteins in cell lysates was measured by anti-phosphotyrosine immunoblotting. TCR stimulation was achieved by incubation for 2 min at 37 °C in the presence of mouse anti-TCR MAb F23.1 plus sheep anti-mouse (SAM) immunoglobulin G (IgG). Lanes 1, 3, 5, 7, 9, 11 and 13: SAM IgG alone (–) lanes and 2, 4, 6, 8, 10, 12 and 14: MAb F23.1 plus SAM IgG (+) lanes. Lanes 1 and 2, Neo.2; 3 and 4, Neo.4; 5 and 6, Csk wt.1; 7 and 8, Csk wt.5; 9 and 10, Csk wt.28; 11 and 12, Csk wt.54; and 13 and 14, F505 Lck-expressing BI-141 cells (F505.9). Exposure, 28 h. **b**, TCR-induced lymphokine production. The release of IL-2 following treatment with anti-TCR MAb F23.1 was measured. Concentrations of antibody are shown on the abscissa (logarithmic scale); incorporation of [³H]thymidine in IL-

2-dependent HT-2 cells is shown on the ordinate (logarithmic scale). c.p.m., Counts per minute. Controls without addition are shown as zero on the abscissa.

METHODS. TCR-induced tyrosine protein phosphorylation was studied as outlined elsewhere^{10,12} using mouse anti-TCR Vβ8 MAb F23.1 (ref. 18). Anti-phosphotyrosine immunoblotting was done as described previously¹⁰, using either affinity-purified polyclonal rabbit anti-phosphotyrosine antibodies (our unpublished data) or the mouse monoclonal anti-phosphotyrosine antibody MAb 4G10 (UBI). Cells expressing activated (F505) Lck polypeptides were reported elsewhere¹⁰. IL-2 production was assayed according to a previously described protocol¹⁰. This assay measures the incorporation of [³H]thymidine in IL-2-dependent HT-2 cells.

Although Csk is abundant in the cytoplasm, some is associated with cellular membranes^{4,6,8}. This localization presumably allows interactions with membrane-bound Src-related enzymes¹³. To examine the effect of Csk on membrane-associated processes more clearly, we evaluated the function of membrane-targeted versions of this enzyme (Fig. 3a). Two different chimaeric proteins were created to ensure that the improved function of these polypeptides was a consequence of membrane targeting. Whereas one chimaera (Src–Csk) had the Src myristylation signal, the other (Csk–Ras) contained the Ha-Ras farnesylation and palmitoylation signals. In both cases, the added sequences are sufficient to target heterologous proteins to the plasma membrane^{14,15}. Constructs encoding either kinase-active or kinase-defective (Lys 222 to Arg 222 mutant (R222)) proteins were produced.

Multiple cell lines expressing these various polypeptides were obtained, as outlined above. As a result of the additional amino-acid sequences, the chimaeric proteins had a slower mobility in SDS–polyacrylamide gels (Fig. 3b; and data not shown). The Src–Csk chimaera was expressed at levels comparable to those of the endogenous p50^{csk} (Fig. 3b, lanes 2–7). All cell lines showed similar levels of TCR, CD3ε, ζ, TCR-associated ζ, p56^{lck}, p59^{fynT}, CD45 and Thy 1 (data not shown). The impact of expression of membrane-targeted Csk on anti-TCR antibody-mediated tyrosine protein phosphorylation was tested (Fig. 3c). Consistent with the results above, expression of Src–Csk greatly reduced the TCR-induced signal (Fig. 3c, lanes 5–10). The kinase-defective version of this polypeptide failed to inhibit substrate phosphorylation (lanes 11–16). A time-course experiment (Fig. 3d) demonstrated that membrane-targeted Csk fully abrogated TCR-mediated substrate phosphorylation. In addition, Src–Csk blocked anti-CD3 antibody (145-2C11)-triggered tyrosine protein phosphorylation (Fig. 3e, lane 6). But incubation of Src–Csk-expressing T cells with the tyrosine phosphatase inhibitors phenylarsine oxide (Fig. 3f, lane 5) or pervanadate (Fig. 3f, lane 6) promptly increased their content of phosphotyrosine-containing proteins, indicating that the chimaeric protein did

not block all tyrosine phosphorylation responses in these cells. Results similar to those observed with Src–Csk were obtained with the Csk–Ras chimaeras (data not shown).

The influence of membrane-targeted Csk on antigen receptor-induced lymphokine production was also measured (Fig. 4). These experiments showed that the two membrane-targeted Csk polypeptides completely abrogated TCR-induced IL-2 production (Fig. 4a). Clearly, the chimaeric molecules were more efficient than wild-type Csk at regulating this TCR-mediated response (an assay comparing cell lines containing similar amounts of total Csk protein is shown in Fig. 4b). It was important that expression of kinase-defective versions of the membrane-targeted Csk proteins did not prevent lymphokine release (Fig. 4c; and data not shown). Finally, we tested the ability of FynT molecules carrying a mutation at the regulatory C-terminal tyrosine residue (Tyr 528 to Phe 528 (F528) mutant) to rescue the uncoupling of TCR signalling by Csk (Fig. 4d). An assay representative of eight independent experiments demonstrated that expression of F528 FynT, but not wild-type FynT or a kinase-defective version of F528 FynT (M296F528 FynT), restored anti-TCR antibody-induced lymphokine release in cells containing the Csk–Ras chimaera.

These results show that the tyrosine protein kinase p50^{csk} negatively regulates TCR-induced tyrosine protein phosphorylation and IL-2 production. This effect was observed with overexpression of Csk or, more efficiently, when Csk expression was targeted to the plasma membrane. Inhibition of TCR signalling was highly dependent on the catalytic activity of p50^{csk}, as indicated by the fact that Csk polypeptides carrying a mutation in the putative phosphotransfer motif did not significantly suppress T-cell responsiveness. Note that neither overexpression of wild-type p50^{csk} nor expression of membrane-targeted versions of Csk significantly elevated tyrosine protein phosphorylation in resting or TCR-stimulated lymphocytes. Thus, Csk seems to function by stimulating the tyrosine phosphorylation of a very limited set of intracellular proteins. Because p50^{csk} efficiently phosphorylates the regulatory C-terminal tyrosine residue of p56^{lck} and

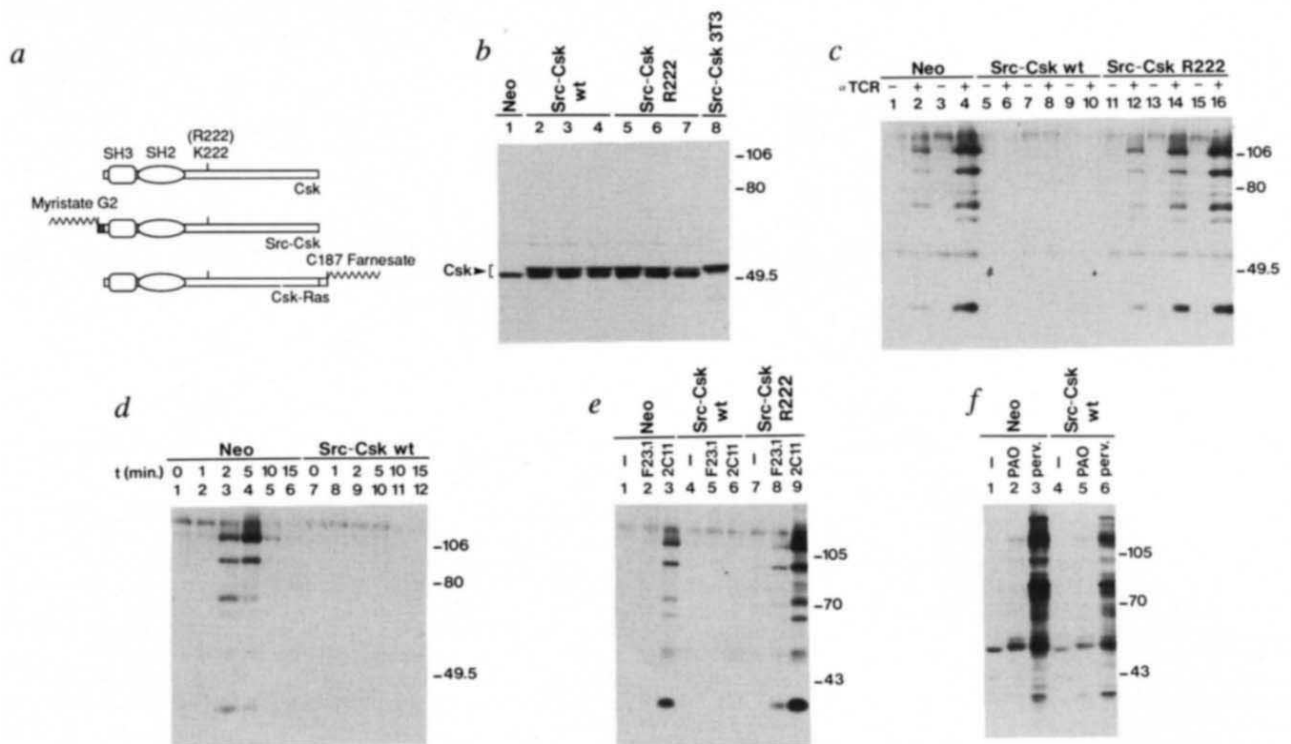


FIG. 3 Effects of membrane-targeting of p50^{Csk} on TCR-induced tyrosine protein phosphorylation. **a**, Csk chimaeras. Schematic representations of Csk, Src-Csk and Csk-Ras are shown. **b**, Expression of Src-Csk. The abundance of Csk in cells containing the neomycin phosphotransferase alone (Neo; lane 1), wild-type Src-Csk (Src-Csk wt; lanes 2–4) or kinase-defective Src-Csk (Src-Csk R222; lanes 5–7) was determined by anti-Csk immunoblotting. Lanes 1, Neo.5; 2, Src-Csk wt.14; 3, Src-Csk wt.39; 4, Src-Csk wt.50; 5, Src-Csk R222.38; 6, Src-Csk R222.40; 7, Src-Csk R222.46; and 8, Src-Csk expressing NIH 3T3 cells. Exposure, 8 h. **c**, TCR-induced tyrosine protein phosphorylation. Cells were stimulated with anti-TCR MAb F23.1, and the abundance of phosphotyrosine-containing proteins measured by anti-phosphotyrosine immunoblotting. Lanes 1, 3, 5, 7, 9, 11, 13 and 15, SAM IgG alone and 2, 4, 6, 8, 10, 12, 14 and 16, MAb F23.1 plus SAM IgG. Lanes 1 and 2, Neo.1; 3 and 4, Neo.2; 5 and 6, Src-Csk wt. 14; 7 and 8, Src-Csk wt.39; 9 and 10, Src-Csk wt.50; 11 and 12, Src-Csk R222.38; 13 and 14, Src-Csk R222.40; and 15 and 16, Src-Csk R222.46. Exposure, 72 h. **d**, Time-course of TCR-induced tyrosine protein phosphorylation. Cells were stimulated with anti-TCR MAb F23.1 for various periods of time before processing for anti-phosphotyrosine immunoblotting. Lanes 1–6, Neo.1 and 7–12, Src-Csk wt.39. Lanes 1 and 7, SAM IgG alone for 1 min; 2 and 8, MAb F23.1 plus SAM IgG for 1 min; 3 and 9, MAb F23.1 plus SAM IgG for 2 min; 4 and 10, MAb F23.1 plus SAM IgG for 5 min; 5 and 11, MAb F23.1 plus SAM IgG for 10 min; and 6 and 12, MAb F23.1 plus SAM IgG for 15 min. Exposure, 72 h. **e**, CD3-induced tyrosine protein phosphorylation. Cells were stimulated with either anti-TCR MAb F23.1 or hamster anti-CD3 MAb 145–2C11. Lanes 1–3, Neo.1; 4–6, Src-Csk wt.39 and 7–9, Src-Csk R222.46. Lanes 1, 4 and 7, SAM IgG alone; 2, 5 and 8, MAb F23.1 plus SAM IgG; and 3, 6 and 9, MAb 145–2C11 plus rabbit anti-hamster (RAH) IgG. The 53K band detected in lanes 3, 6 and 9 is the heavy chain of RAH IgG. Exposure, 24 h. **f**, Effects of treatment with tyrosine phosphatase inhibitors on intracellular tyrosine protein phosphorylation. Cells were incubated for 10 min at 37 °C with

either phenylarsine oxide or pervanadate. Lanes 1–3, Neo.1 and 4–6, Src-Csk wt.39. Lanes 1 and 4, untreated control; 2 and 5, phenylarsine oxide and 3 and 6, pervanadate. Exposure, 72 h. **METHODS.** The *src-csk* cDNA was generated by in-frame ligation of a *HindIII*–*NaeI* fragment from the avian *c-src* cDNA¹⁹ (encoding the first 15 amino acids of p60^{c-src}) with an *Eco47III*–*HindIII* fragment from the *csk* cDNA⁴ (which includes the entire coding sequence of *csk*, except for the initiating methionine). The *Eco47III* site was artificially introduced in the *csk* cDNA through polymerase chain reactions (PCRs)²⁰, with the oligonucleotide 5' CTCAGAAGCGCTCGGCTAT 3'. To create the *csk-ras* cDNA, a *SacI*–*XbaI* fragment encoding the last 18 amino acids of human p21^{Ha-ras} (produced by PCRs using the human *Ha-ras* gene from pEJ6.6 (ref. 21), and the oligonucleotides 5' AGCTGCGGGAGCTCAACCTCC 3' and 5' TCCATGTCTAGAGCTTGT 3') was ligated in-frame to a *HindIII*–*SacI* fragment from the *csk* cDNA. This *SacI* site was generated by PCR using the oligonucleotide 5' CACAGGTGGAGCTCATG 3'. The resulting DNA construction encompasses the entire coding sequence of *csk*, except for the two most C-terminal residues, which lie outside the catalytic domain. The lysine residue of the putative phosphotransfer motif of p50^{Csk} (K222) was mutated to arginine (R222) through site-directed mutagenesis²² with the oligonucleotide 5' TTAATGCATCTGACAGCTACTTTTGT 3'. A similar mutation in numerous protein kinases greatly reduces catalytic activity²³. Kinase-defective chimaeric cDNAs were generated through standard recombinant DNA technology. Immunoblotting was as outlined in the legends to Figs 1 and 2. Antibody-mediated stimulation was as described for Fig. 2. When hamster anti-CD3ε MAb 145–2C11 (ref. 24) was used for activation, rabbit anti-hamster (RAH) IgG was added as second-step antibody. To test the effect of tyrosine phosphatase inhibitors on intracellular tyrosine protein phosphorylation, cells were incubated for 10 min at 37 °C in the presence of either 35 μM phenylarsine oxide²⁵ (Sigma) or premixed 0.1 mM Na₃VO₄ plus 10 mM H₂O₂ (pervanadate²⁶), before cell lysis and processing for anti-phosphotyrosine immunoblotting.

p59^{FynT} *in vitro*, and because F528 FynT rescued the negative effect of membrane-associated Csk on TCR-induced lymphokine release, Csk probably functions *in vivo* by acting directly on FynT and/or Lck. Our studies indicate that the extent of C-terminal tyrosine phosphorylation of Lck and FynT in resting

T cells is not significantly augmented by overexpression of Csk or expression of membrane-targeted versions of Csk (unpublished data). Hence, transient Csk-induced phosphorylation may be sufficient to repress effectively the catalytic function of Src family members *in vivo*. Alternatively, and especially in T lymphocytes,

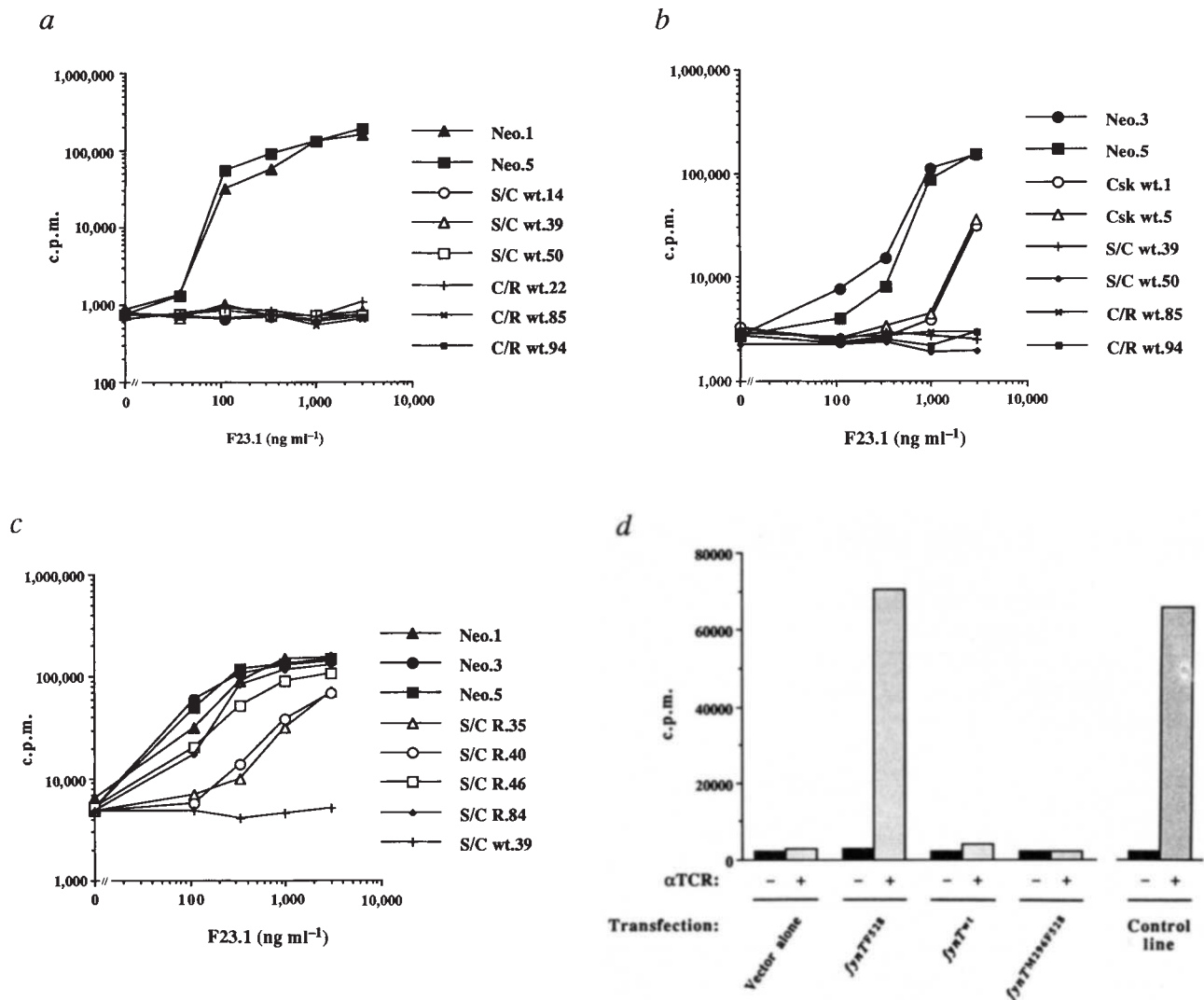


FIG. 4 Effects of membrane-targeting of p50^{Csk} on TCR-induced lymphokine production. *a*, Effects of Src-Csk and Csk-Ras. S/C, Src-Csk; C/R, Csk-Ras. *b*, Comparison of the effects of wild-type Csk and membrane-targeted versions of Csk. wt, Wild-type; S/C, Src-Csk; C/R, Csk-Ras. *c*, Effect of kinase-defective Src-Csk and Csk-Ras. S/C, Src-Csk; C/R, Csk-Ras. *d*, Effect of coexpression of F528 FynT on the uncoupling of TCR signalling by membrane-targeted Csk. Cells expressing Csk-Ras (C/R wt.85) were transfected with DNAs encoding F528 FynT, wild-type FynT or a kinase-defective version of F528 FynT (M296F528 FynT), or with the vector plasmid alone, and were tested for anti-TCR MAb F23.1-induced lymphokine production. The 'control line' expresses the neomycin phosphotransferase alone (Neo.5; the number of Neo.5 cells used in the assay was reduced based on the transfection efficiency in C/R wt.85 cells, that is 2–5%). c.p.m., Counts per minute; –, medium alone; +, MAb F23.1.

METHODS. Cells were tested for MAb F23.1-induced lymphokine

release, as described in the legend to Fig. 2. For coexpression of the various FynT polypeptides in cells expressing the Csk-Ras chimera, 5×10^6 cells were transfected with DEAE-dextran (Pharmacia; 500 μ g ml⁻¹ for 30 min) and plasmid DNAs (5 μ g) encoding F528 FynT, wild-type FynT or a kinase-defective version of F528 FynT (M296F528 FynT), or the vector plasmid alone, as described²⁷. The constructs used were reported elsewhere¹², and allow comparable levels of p59^{FynT} expression in stable BL-141 T-cell transfectants¹². After transfection, cells were incubated for 24 h in serum-containing medium, and then tested for anti-TCR MAb F23.1-induced IL-2 production, as described for Fig. 2. Assays were done in triplicate, using MAb F23.1 at a concentration of 3,000 ng ml⁻¹. Transfection efficiency was monitored by parallel transfection with *lacZ* DNA. After 24 h, *lacZ*-expressing cells were revealed chemically²⁸. It was consistently observed that 2–5% of cells acquired the characteristic green appearance (data not shown).

it is possible that only the fraction of Src-related molecules participating in a given signalling response is actively regulated by Csk. □

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Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25

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NEUROTRANSMITTER release is potently blocked by a group of structurally related toxin proteins produced by *Clostridium botulinum*¹. Botulinum neurotoxin type B (BoNT/B) and tetanus toxin (TeTx) are zinc-dependent proteases that specifically cleave synaptobrevin (VAMP), a membrane protein of synaptic vesicles^{2,3}. Here we report that inhibition of transmitter release from synaptosomes caused by botulinum neurotoxin A (BoNT/A) is associated with the selective proteolysis of the synaptic protein SNAP-25. Furthermore, isolated or recombinant L chain of BoNT/A cleaves SNAP-25 *in vitro*. Cleavage occurred near the carboxyterminus and was sensitive to divalent cation chelators. In addition, a glutamate residue in the BoNT/A L chain, presumably required to stabilize a water molecule in the zinc-containing catalytic centre, was required for proteolytic activity. These findings demonstrate that BoNT/A acts as a zinc-dependent protease that selectively cleaves SNAP-25. Thus, a second component of the putative fusion complex mediating synaptic vesicle exocytosis is targeted by a clostridial neurotoxin.

Intact nerve terminals (synaptosomes) were used to correlate inhibition of transmitter release by BoNT/A with changes in synaptic protein patterns. Synaptosomes were incubated with 150 nM BoNT/A for 90 min before transmitter release was initiated by KCl-depolarization. Glutamate release was monitored with a photometric assay⁴. Figure 1a shows that preincubation in the presence of 150 nM BoNT/A led to a significant inhibition of neurotransmitter release ($69.2 \pm 1.2\%$). Heat-inactivated

BoNT/A or isolated BoNT/A-L chain had no effect (not shown).

In a search for BoNT/A target proteins, we screened a large number of antibodies directed against synaptic proteins. Although none of these antigens appeared to be affected by BoNT/A, we noticed that with one of the sera a band of M_r roughly 25K was degraded which was also recognized by the corresponding preimmune serum. This prompted us to investigate whether this band was identical with SNAP-25 (synaptosomal associated protein of 25K), a membrane-associated protein of the same M_r that is enriched in synaptic plasma membranes⁵. Using an antibody raised against a C-terminal peptide of SNAP-25⁵, we found that BoNT/A caused a significant reduction ($52 \pm 3\%$) in the amount of SNAP-25, corresponding to the inhibition of glutamate release, without affecting HPC-1/syntaxin⁶, neuexin I⁷, or synaptic vesicle proteins⁸ such as synapsin I, synaptotagmin, synaptophysin, rab3a, p29, SV2 (Fig. 1b and not shown). Similarly, no consistent changes were seen in the level of synaptobrevin although occasionally a slight reduction was observed. In addition, no nonspecific breakdown of other proteins was visible (Fig. 1b, Coomassie blue staining). We then affinity-purified the antibody from the preimmune serum that recognized the 25K band. We confirmed, by two-dimensional electrophoresis, that this band indeed corresponded to SNAP-25 (not shown). With this antibody, an immunoreactive fragment of only slightly reduced M_r was discovered after BoNT/A-treatment, which was not seen with the antibody reacting with the C terminus of SNAP-25 (Fig. 1b, bottom). The decrease of immunoreactivity of the full-length protein with this affinity-purified antibody was 59% (using a shorter exposure of the same blot for quantifying).

To evaluate whether cleavage of SNAP-25 is due to a direct interaction of the toxin with the substrate, we incubated a synaptic membrane fraction with BoNT/A that was pretreated with dithiothreitol to release the L chain. BoNT/A led to a reduction in the amount of SNAP-25 (Fig. 2; decrease in immunoreactivity a, 75% and b, 70%, respectively). No changes were observed in other synaptic proteins or in the overall protein pattern (Fig. 2 and not shown), demonstrating the specificity of the proteolysis. Again, a fragment was detected with the SNAP-25 antibody derived from our preimmune serum which migrates at a slightly reduced M_r . This suggests that cleavage occurs at the same position as it does in intact nerve terminals.

To analyse SNAP-25 cleavage independent of epitope-dependent antibodies and without interference by other synaptic proteins, we translated SNAP-25 *in vitro* in the presence of [³⁵S]methionine. As shown in Fig. 3 (left lane), a major translation product with an apparent M_r of 25K was generated that comigrated with the native protein. When the translation product was incubated with reduced BoNT/A, it was converted completely into a band of higher mobility (apparent M_r -shift $\sim 1,000$ –2,000) that comigrated with the fragments shown in Figs 1 and 2. Under the same conditions, neither HPC-1/syntaxin nor synaptotagmin (Fig. 3) or synaptobrevin (not shown) were affected even when the amount of toxin was increased and the incubation time extended. The chelators 1,10-phenanthroline (PTL) and 2,2'-dipyridyl (DPD) largely prevented proteolysis indicating that BoNT/A acts as a metal-dependent protease. In contrast, 10 mM EDTA did not inhibit proteolysis, suggesting a high affinity of the metal cation binding site.

Using SNAP-25 translated *in vitro* as substrate, we investigated whether in analogy to tetanus toxin (TeTx) the L chain of BoNT/A is sufficient for proteolytic activity or whether an interaction with the H chain is required. As shown in Fig. 4, (right lanes), purified BoNT/A L chain was as active as the reduced form of the holotoxin. In contrast, purified H chain did not cause any breakdown. To rule out any interference with contaminating proteases, BoNT/A L chain was also generated from cloned DNA⁹ by *in vitro* translation in the absence of radioactive tracer and tested for activity. In addition, recombi-

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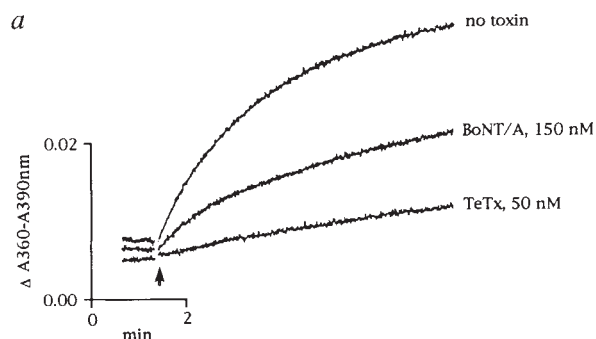
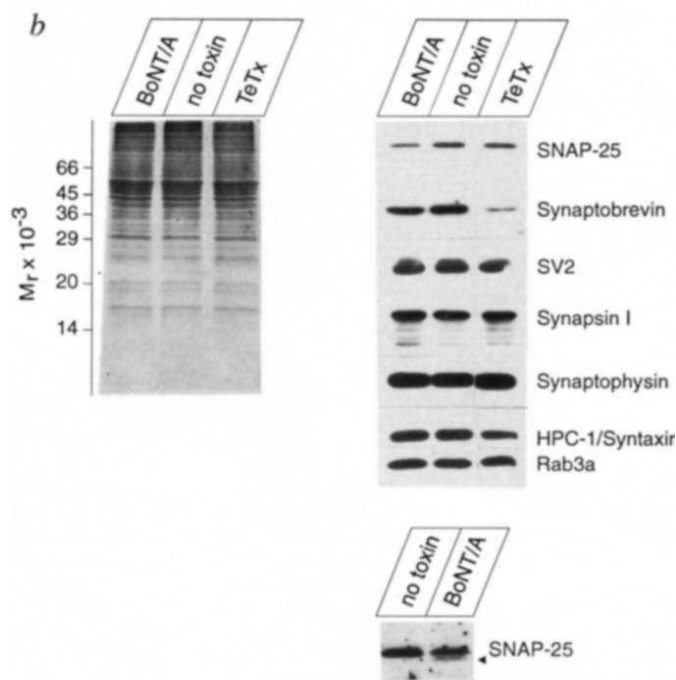


FIG. 1 Inhibition of neurotransmitter release by BoNT/A from isolated nerve terminals is associated with cleavage of SNAP-25. **a**, BoNT/A inhibits glutamate release from isolated nerve terminals. Release was measured spectrophotometrically by following the oxidation of glutamate by glutamate dehydrogenase. The arrow indicates addition of 50 mM KCl (final concentration). All traces are corrected for baseline shifts associated with the addition of KCl. Note that inhibition of glutamate release by BoNT/A is not as complete as for TeTx which was used as control. **b**, SNAP-25 is selectively degraded in the presence of BoNT/A. At the end of the photometric recording (**a**), the synaptosomes were analysed by SDS-PAGE and immunoblotting (20 μ g protein per lane, 12% gels). Left, Coomassie blue staining; right, immunoblots. Immunoblotting for SNAP-25 was done either with an antibody generated against a synthetic peptide corresponding to the C-terminal 12 amino acids⁵ (top) or with an affinity-purified antibody obtained from a preimmune serum (bottom, see text). The arrowhead points to a cleavage product of SNAP-25. To control for breakdown during sample processing, toxin was added at the end of the photometric recording. No cleavage was observed under these conditions. Note that synaptobrevin is cleaved by TeTx^{2,3} whereas SNAP-25 remains unaffected.

METHODS. Preparation and toxin incubation of synaptosomes was done precisely as described previously, with a 90-min preincubation period before stimulation for 8 min^{3,4,17}. Glutamate release was monitored as described⁴. In the experiments shown here and in Fig. 2, the following antibodies were used for immunoblotting: SNAP-25 (kindly provided by M. C. Wilson, La Jolla)⁵, synaptobrevin¹⁸, synaptotagmin¹⁹, synaptophysin²⁰, SV2 (gift of K. Buckley, Boston)²¹. HPC-1/syntaxin (gift



of C. Barnstable, New Haven)²² and rab3A²³. A rabbit serum against synapsin I/II was raised using a N-terminal peptide sequence common to all isoforms as the antigen (T.C.S., unpublished results). SDS-PAGE was done according to standard procedures²⁴ using the Bio-Rad Protean II minigel system. Immunoblotting and visualization with ¹²⁵I-labelled protein A was as described²¹. The bottom panel was visualized with the enhanced chemiluminescence method (Amersham) following the manufacturers instructions. Protein concentrations were determined according to Bradford²⁵. The SNAP-25 antibody derived from a preimmune serum was affinity-purified using electrophoretically separated synaptic membranes²⁶. BoNT/A was produced and purified from the Hall strain of *Clostridium botulinum* type A²⁷.

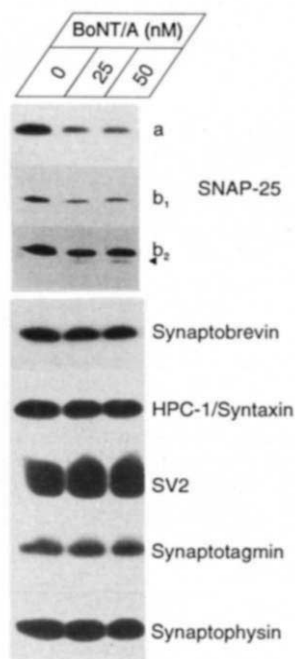


FIG. 2 BoNT/A selectively cleaves SNAP-25 when incubated with isolated synaptic membranes. Membranes were incubated for 60 min at 37 °C with increasing concentrations of BoNT/A that was reduced with dithiothreitol (10 mM). The samples were analysed for SNAP-25 by SDS-PAGE and immunoblotting as described in Fig. 1 using both a C-terminal-specific antibody⁵ (**a**) and an antibody affinity-purified from a preimmune serum (**b**₁ and **b**₂). **b**₂ shows the same blot as **b**₁ but exposure time was fivefold longer in order to visualize the breakdown product (arrowhead).

METHODS. A membrane fraction was isolated by hypotonic lysis of synaptosomes¹⁷ followed by sedimentation of the membranes for 10 min at 12,000g. Incubation was for 60 min at 37 °C in 20 mM HEPES-NaOH, pH 7.0, 100 mM NaCl, 10 mM dithiothreitol, 0.05% Triton X-100. For electrophoresis and immunoblotting see legend to Fig. 1.

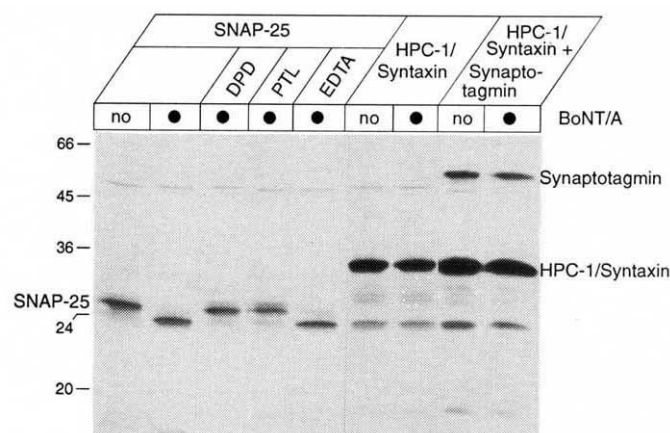


FIG. 3 BoNT/A cleaves SNAP-25 translated *in vitro* and requires divalent metal cations as cofactors. For comparison, HPC-1/syntaxin and synaptotagmin I were translated in parallel incubations. Translation was done in the presence of [35 S]methionine to label the products. All samples were analysed by SDS-PAGE and fluorography. The chelator concentrations were: 20 mM DPD, 20 mM PTL and 10 mM EDTA. The numbers on the left indicate $M_r \times 10^{-3}$. During translation of HPC-1/syntaxin DNA, an additional product is generated that comigrates with the SNAP-25 fragment. Abbreviations, DPD, 2,2'-dipyridyl; PTL, 1,10-phenanthroline.

METHODS. cDNA for synaptotagmin I was described previously²⁸. SNAP-25 was cloned by PCR from mouse brain cDNA. The identity of the clone was confirmed by restriction mapping. Clones for syntaxin A (lacking the first three amino acids) and syntaxin B (lacking the first four amino acids) were provided by M. K. Bennet and R. H. Scheller (Stanford, CA)⁶. The 5' ends were filled with appropriately designed PCR-primers that contained an EcoRI restriction site. Transcription of all cDNAs were placed under control of a T7 or T3 promoter by subcloning into CDM8²⁹ that was modified to contain EcoRI sites in the polylinker or pBluescript SKII+³⁰. For transcription-translation, the TnT-system was used (Promega, Madison, WI). DNA (0.5 μ g) was incubated with T7 or T3 polymerase in a reaction mixture of 25 μ l containing [35 S]methionine following the instructions of the manufacturer. Toxin incubations were as in Fig. 2.

nant L chain variants of BoNT/A were constructed that contained point mutations in the Glu-residue Glu₂₂₄. In other zinc-dependent metalloproteases, the corresponding glutamate serves to coordinate a hydrogen-bonded water molecule in one of the four tetrahedral positions around the catalytic zinc ion¹⁰. As shown in Fig. 4 (left lanes), the wild-type form of BoNT/A-L chain efficiently cleaved SNAP-25, whereas the two L chain mutants in which Glu₂₂₄ was replaced by Gln and Lys, respectively, were inactive. Similarly, the recombinant TeTx L chain that caused breakdown of synaptobrevin in parallel incubations (not shown) did not cleave SNAP-25. Together, these data demonstrate that BoNT/A L chain operates as a Zn^{2+} -dependent protease that selectively cleaves SNAP-25. Because an N-terminally truncated, but not a C-terminally truncated mutant form of SNAP-25 is cleaved (not shown), it appears that the cleavage site is localized close to the C terminus of the molecule.

Although it cannot be excluded that BoNT/A has additional targets in the synapse, it is likely that the cleavage of SNAP-25 is primarily responsible for the inhibition in neurotransmitter release. Thus the target of BoNT/A is different from that of TeTx and BoNT/B which cleave synaptobrevin^{2,3}. This agrees with differences observed in their physiological action. Although BoNT/A poisoning results in a stronger depression of spontaneous and evoked release than poisoning by BoNT/B or TeTx, the inhibition can be more easily counteracted by 4-aminopyridine¹¹. In addition, black widow spider venom, a neurotoxin that elicits massive exocytosis at the neuromuscular endplate, causes exocytosis of synaptic vesicles from BoNT/A but not from BoNT/B or TeTx-poisoned synapses¹².

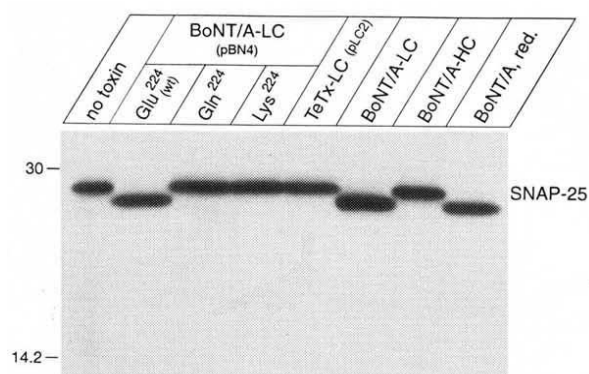


FIG. 4 The L chain of BoNT/A cleaves SNAP-25 and requires Glu 224 for activity. In the experiments shown in the left part of the figure, SNAP-25 was translated *in vitro* in the presence of [35 S]methionine and combined at the end of the incubation with BoNT/A-L chain and TeTx-L chain that were translated *in vitro* in parallel incubations using unlabelled amino acids. In the experiments shown in the right three lanes, BoNT/A components were used that were purified from bacteria. Note that neither the H chain nor the two mutant L chains or TeTx L chains caused cleavage of SNAP-25.

METHODS. For the construction of the Gln 224 and Lys 224 mutants, two new singular restriction sites, BamHI and NruI were generated by PCR in positions 1,002 and 1,063⁹. The sites flank the sequence coding for a conserved HExxHxxH motif that is essential for Zn^{2+} binding, thus allowing replacement of the internal sequence by appropriate synthetic oligonucleotides. *In vitro* translation was as given in Fig. 3 but linearized DNA was used as template for the generation of mRNA. SNAP-25 mRNA was translated in the presence of [35 S]methionine, BoNT/A-LC mRNA was translated in an unlabelled form, both under control of SP6 promoter in pSP72 (Promega, Madison, WI). At the end of the translation period (60 min, 30 °C), cycloheximide was added to both translation assays (250 μ M final concentration) before mixing of the samples. Incubation was for 2 h at 37 °C. Electrophoresis and fluorography were as described in Fig. 3.

SNAP-25 is a highly conserved membrane protein¹³ that has been speculated earlier to be involved in exocytosis⁵. It is post-translationally modified by palmitoyl-side chains that presumably mediate membrane-binding^{5,14}. Recently, SNAP-25 has been shown to be part of a protein complex that interacts in an ATP-dependent manner with soluble proteins factors, NSF and SNAPs (SNAP, in this case, is an acronym for soluble NSF attachment proteins)¹⁵. NSF and SNAPs are required for intracellular membrane fusion events and are highly conserved in evolution¹⁶. The other components of the complex are the vesicle protein synaptobrevin and the synaptic membrane protein HPC-1/syntaxin¹⁵. These findings suggest that these three membrane proteins may form the core of an exocytotic membrane fusion complex that requires NSF and SNAPs as cofactors and that may be further controlled by other proteins such as synaptotagmin and neurexin. This view is now strongly supported by the findings that two members of this complex are the specific targets of related clostridial neurotoxins, with synaptobrevin being the target of BoNT/B and TeTx^{2,3} and SNAP-25, as demonstrated in this study, being the target of BoNT/A. It appears that the clostridial neurotoxins have evolved as molecules that recognize the synaptic fusion machine whereby individual toxins target different components of the complex. □

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Dynamin GTPase regulated by protein kinase C phosphorylation in nerve terminals

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DYNAMIN is a microtubule-binding protein with a microtubule-activated GTPase activity^{1–3}. The gene encoding dynamin is mutated in *shibire*^{4,5}, a *Drosophila* mutant defective in endocytosis in nerve terminals and other cells^{6–9}. These observations place dynamin into two distinct functional contexts, suggesting roles in microtubule-based motility or in endocytosis. We report here that dynamin is identical to the neuronal phosphoprotein dephosphin (P96), originally identified by its stimulus-dependent dephosphorylation in nerve terminals^{10–13}. Dynamin is a protein doublet of *M_r* 94 and 96K arising by alternative splicing of its primary transcript. In the nerve terminal, both forms of dynamin are phosphorylated by protein kinase C (PKC) and are quantitatively dephosphorylated on excitation. *In vitro*, dynamin is also phosphorylated by casein kinase II which inhibits PKC phosphorylation. Phosphorylation by PKC but not by casein kinase II enhances the GTPase activity of dynamin 12-fold. The dynamins are therefore a group of nerve terminal phosphoproteins whose GTPase is regulated by phosphorylation in parallel with synaptic vesicle recycling. The regulation of dynamin GTPase could serve as the trigger for the rapid endocytosis of synaptic vesicles after exocytosis.

In isolated nerve terminals, dephosphin (also called P96) is a

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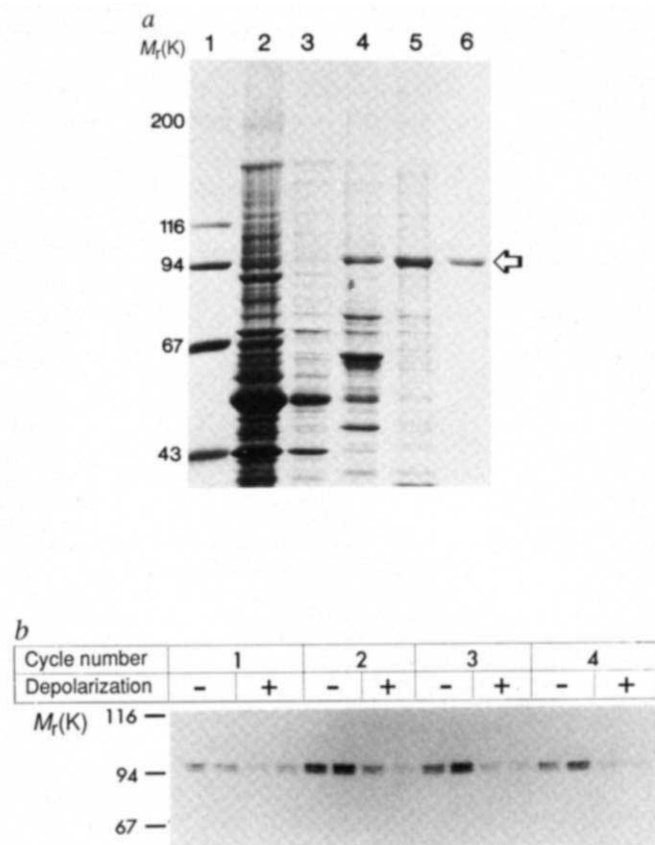


FIG. 1 *a*, Purification of dephosphin from rat brain. Fractions from different stages of the purification of dephosphin were analysed by SDS-PAGE and Coomassie blue staining. Lane 1, *M_r* standards; lane 2, total brain (90 µg protein); lane 3, 150 mM NaCl extract (40 µg); lane 4, pooled peak fractions from S-Sepharose column (40 µg); lane 5, pooled peak fractions from hydroxy-apatite column (40 µg); lane 6, pooled peak fractions from mono-Q column (1.5 µg). *b*, Phosphorylation of dynamin/dephosphin in ³²P-labelled synaptosomes. In experiments done in duplicate, synaptosomes were labelled with inorganic ³²P and subjected to multiple cycles of depolarization (10 s in 41 mM KCl) and repolarization (5 min in 4.7 mM KCl). After each step, dynamin/dephosphin was immunoprecipitated from the synaptosomes and analysed by SDS-PAGE and autoradiography.

METHODS. *a*, Rat brains (70 g) were homogenized in 0.4 l buffer A (20 mM NaH₂PO₄ pH 7.0, 10 mM DTT, 17 mg l⁻¹ leupeptin and 1 mM PMSF) containing 1 mM CaCl₂ and centrifuged for 30 min at 10,000g. The pellet was washed with buffer A containing 0.1 mM CaCl₂, buffer A containing 5 mM EGTA and 2 mM EDTA, and buffer A containing 0.15 M NaCl. The last supernatant (lane 3) was dialysed against buffer A and chromatographed on S-Sepharose with a 0.0 to 0.4 M NaCl gradient in buffer A containing 0.1% Triton X100. Peak fractions were pooled (lane 4), dialysed against 20 mM KH₂PO₄ pH 7.0, 1 mM EDTA, 0.1% Triton X100, and loaded onto a hydroxy-apatite column that was eluted with a 20 to 350 mM KH₂PO₄ gradient. Pooled peak fractions (lane 5) were dialysed against 20 mM Tris-HCl pH 7.7, 1 mM DTT, and 0.1% Triton X100 and chromatographed on a mono-Q column with a 0.0 to 0.3 M NaCl gradient, resulting in nearly homogeneous dephosphin (lane 6, arrow). Dephosphin was assayed during the purification by phosphorylation with exogenous PKC and phosphopeptide mapping. *b*, Synaptosomes labelled with inorganic ³²P (refs 10, 11) were depolarized by addition of KCl (final concentration 41 mM) and repolarized by centrifugation (1 min at 10,000g) and resuspension in buffer containing 4.7 mM KCl. Aliquots were removed at each time point, centrifuged as above, and the pellet was resuspended in 0.1 ml lysis buffer (25 mM Tris-HCl pH 7.4, 1% Triton X100, 150 mM NaCl, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 mM O-phenanthroline, 1 g l⁻¹ bacitracin, and 20 mg l⁻¹ leupeptin) and frozen. Immunoprecipitations were done using standard protocols¹⁶ with antibodies raised in rabbits to purified dynamin/dephosphin (lane 6, *a*). Similar results were also obtained with anti-peptide antibodies to dynamin.

TABLE 1 Sequences of tryptic peptides from purified dephosphin: comparison with dynamin

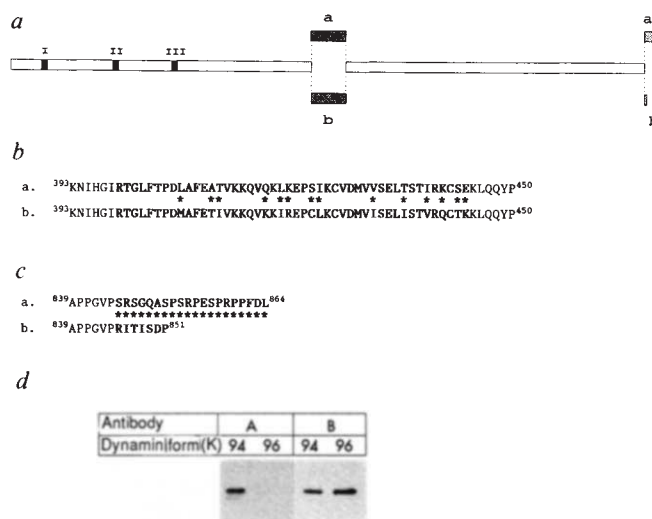
Peptide	Origin of peptide	Amino-acid sequence	Residue number in cDNA sequences
1A	Dephosphin	(K) SSVL	44–48
	Dynamin	K SSVL	
1B	Dephosphin	(K) KFT	89–92
	Dynamin	K KFT	
2	Dephosphin	(K) GISPVPINLR	113–123
	Dynamin	K GISPVPINLR	
3A	Dephosphin	(R) TGLFTPDFAFEATVKK	399–415
	Dynamin	R TGLFTPDFAFEATVKK	
3B	Dephosphin	(K) ?V?MVVSELTSTI	426–439
	Dynamin	K CVDMMVSELTSTI	
4A	Dephosphin	(R) TKEQVMLLID?E	470–482
	Dynamin	R TKEQVMLLIDIE	
5	Dephosphin	(R) QLELACETQEEVD SW KA?FL	601–621
	Dynamin	R QLELACETQEEVD SW KASFL	
4B	Dephosphin	(R) MYHALKEALSIIIGDINT	730–747
	Dynamin	R MYHALKEALSIIIGDINT	
6	Dephosphin	(R) SGQA?PS?PE	846–856
	Dynamin	R SGQASPSRPE	

Purified dephosphin was subjected to SDS-PAGE, blotted onto PVDF membranes¹⁵, and cleaved with trypsin. Tryptic peptides were purified by a single separation by reversed-phase HPLC (peptides 1A, 1B, 3A, 3B, 4A and 4B) or by two successive reversed-phase HPLC separations in different mobile phases (peptides 2, 5 and 6) and sequenced in an ABI amino-acid sequencer. Peptides 1, 3 and 4 resulted in mixed sequences that could be assigned in the rat dynamin sequence¹ as shown as 1A, 1B and so on. The peptides purified by two successive separations resulted in unequivocal sequences. Unreadable residues are marked by question marks, and questionable signals by italics. Residues in parentheses represent amino acids preceding the sites of trypsin cleavage not observed during amino-acid analysis. Peptide sequence 6 could not be assigned in the published sequence but was found in the predicted alternatively spliced C terminus of our cDNA clones (Fig. 2c).

major phosphoprotein that is quantitatively dephosphorylated on depolarization and rephosphorylated at rest^{10–13}. Dephosphin was purified from rat brain using *in vitro* phosphorylation by PKC and phosphopeptide mapping as an assay (Fig. 1a). Tryptic peptides from purified dephosphin were isolated by high-performance liquid chromatography (HPLC) and sequenced. The resulting amino-acid sequences (Table 1) revealed identity of dephosphin with dynamin, a GTP- and microtubule-binding protein that contains a very high microtubule-activated GTPase activity^{1–3}. Eight of the nine peptide sequences from dephosphin were identified in the dynamin sequence¹. The ninth derived from an alternatively spliced variant of dynamin (see below).

Antibodies were raised against purified dephosphin/dynamin and used for immunoprecipitations from ³²P-labelled synaptosomes. The antibodies immunoprecipitated synaptosomal proteins of 94 and 96K that were phosphorylated in synaptosomes and dephosphorylated on depolarization (Fig. 1b). This confirms that the protein we purified is dephosphin, which therefore, based on the amino-acid sequences, is identical with dynamin. Dynamin dephosphorylation in synaptosomes is rapid (<1 min, ref. 11) and closely associated with neurotransmitter release (data not shown, and ref. 12). Rephosphorylation of dynamin occurs more slowly than dephosphorylation (>2 min) and is mediated by PKC¹³. Multiple de- and rephosphorylation cycles

FIG. 2 Alternative splicing of dynamin elucidated by cDNA cloning. a, Relative positions of the two alternatively spliced sequences in dynamin that result in four different forms. The bar diagram depicts dynamin; the alternatively spliced regions are labelled a and b. The positions of the three consensus sequence motifs for GTP-binding sites are numbered I to III. b, Sequences of the differentially spliced middle region of dynamin. The amino-acid sequences of the region in which independent cDNA clones exhibited two different nucleotide sequences are shown in bold type, with nonidentical residues marked by asterisks. Residue numbers of the dynamin sequences¹ are shown at the beginning and end of each segment. c, Amino-acid sequences of the two alternative C termini of dynamins. The alternatively spliced residues are shown in bold type, and residue numbers are indicated at the beginning and end of each segment. Nonidentical residues are marked by asterisks. d, Differential recognition of the two size forms of dynamin by different antibodies. Dynamin isoforms were excised from a long polyacrylamide gel which separated the size variants and loaded into separate lanes of a minigel. Blots of this minigel were then probed with antibodies to a synthetic peptide from the shorter form of dynamin (antibody A) or to purified dynamin (antibody B). Antibodies to the interior peptide from dynamin gave the same results as antibody B (not shown). METHODS. cDNA clones were isolated from a rat brain cDNA library¹⁷ using specific oligonucleotides derived from the rat dynamin sequence¹. The six largest cDNA clones were analysed by DNA sequencing, and all four possible combinations of splice variants were observed in the cDNA clones. For the detection of dynamin isoforms by different antibodies, the two size forms of purified dynamin were separated from each other by SDS-PAGE on 20-cm gradient gels (7.5 to 15%, 1 µg protein per lane), excised from Coomassie blue-stained dried gels and placed in separate wells of a 10% minigel. After electrophoresis and immunoblot-



ting, immunoreactive bands were detected by ECL (Amersham). The antibodies were raised in rabbits either against purified dynamin or against two different peptides coupled to keyhole limpet haemocyanin¹⁸. The two peptides contained dynamin residues 607 to 624 (interior peptide, not shown) and 840 to 851 of the b-variant (C-terminal peptide).

could be done (Fig. 1b), demonstrating efficient cycling of the phosphorylation state of the protein in concert with the functional state of the synaptosomes. Together these results establish that dynamin is one of the major phosphoproteins in brain and is dephosphorylated on nerve terminal depolarization.

Dynamin and dephosphin were identified as protein doublets (Fig. 1b, refs 10, 14) whereas the rat dynamin complementary DNA sequence predicts a single-sized protein¹. We therefore investigated the basis for dynamin heterogeneity in rat brain by complementary DNA cloning. Sequencing of multiple overlapping cDNA clones revealed two positions of alternative splicing as judged by the presence or absence of sequences in multiple independent clones (Fig. 2). The first alternatively spliced segment contained either one of two sequences with identical sizes but distinct nucleotide sequences, suggesting usage of alternative exons. There are 14 amino-acid substitutions in this region, 7 of which are conservative (Fig. 2b). In the second alternatively spliced region, two different C-termini were observed, consisting of 20 and 7 amino acids, respectively (Fig. 2c). The *Drosophila* dynamin cDNAs are also alternatively spliced at a similar position, although there is little sequence homology between the vertebrate and insect messenger RNAs in this region (Fig. 2 and refs 4, 5). All four possible splice combinations were observed in the sequenced rat cDNA clones and appear to be conserved in bovine dynamin (J.M.S. and T.C.S., unpublished data) but

the functional significance of the multiple forms of dynamin is currently unclear.

Only the C-terminal alternative splicing of dynamin creates proteins of different sizes, suggesting that the observed 94/96K protein doublet is due to this event. To test this hypothesis, antibodies were raised to a synthetic peptide containing the shorter C terminus of dynamin (Fig. 2c; residues 840–851) and, as a control, to a peptide from the interior of dynamin (residues 607–624). These antibodies immunoprecipitated dephosphin from brain and strongly reacted with purified protein on immunoblots (data not shown). The antibodies to the purified protein and to the interior peptide reacted with both size forms of dynamin whereas the antibody to the peptide from the shorter C terminus reacted only with the 94K protein, confirming that the two forms arise by C-terminal alternative splicing (Fig. 2d and data not shown).

Purified dynamin is a good substrate *in vitro* for PKC and for casein kinase II but not for cAMP- and cGMP-dependent kinase (Fig. 3a). After *Staphylococcus aureus* V8 cleavage of dynamin phosphorylated by casein kinase II, a single phosphopeptide is observed, whereas PKC phosphorylation gives rise to two phosphopeptides, suggesting that the two kinases phosphorylate dynamin at different sites (Fig. 3b). Phosphopeptide mapping of dynamin phosphorylated in synaptosomes results in a picture identical to that of dynamin phosphorylated by PKC *in vitro*, suggesting that dynamin is primarily phosphorylated by PKC *in*

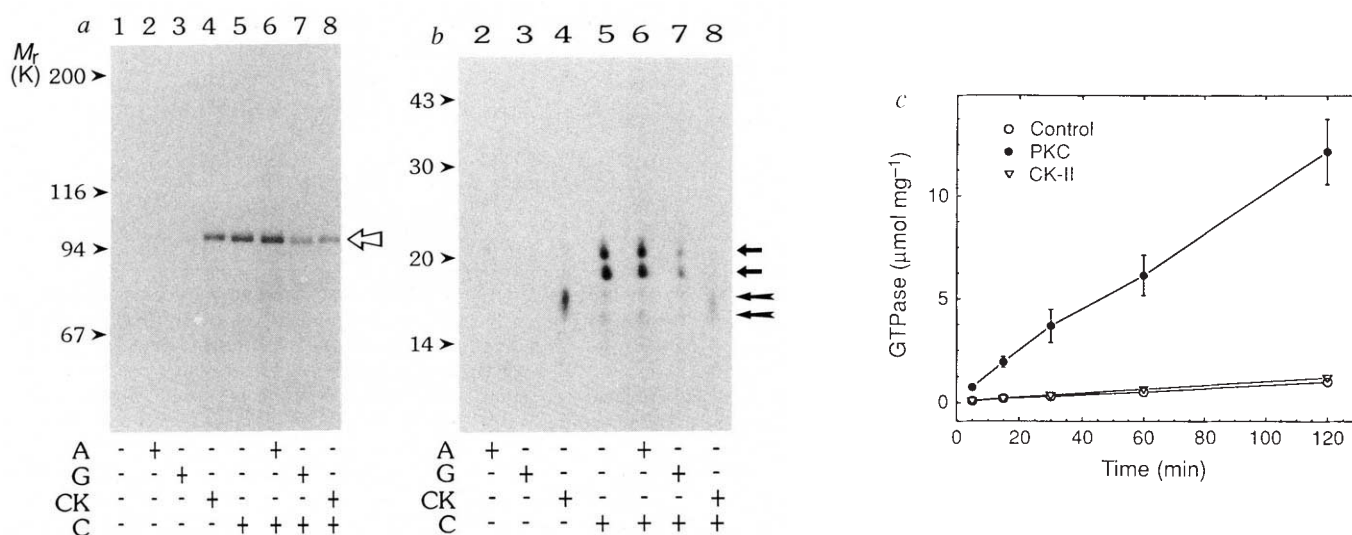


FIG. 3 Analysis of dynamin phosphorylation by different protein kinases: effect on GTPase activity. a, Purified dynamin was subjected to autophosphorylation (lane 1) or to phosphorylation by the catalytic subunit of cAMP-dependent protein kinase (A, lane 2), cGMP-dependent protein kinase (G, lane 3), casein kinase II (CK, lane 4), and PKC (C, lane 5). In lanes 6 to 8, dynamin was coincubated with PKC and each of the other kinases as indicated. b, Phosphopeptide maps of dynamin phosphorylation sites after cleavage with *S. aureus* V8 protease. Samples in each lane were excised from the same lane number shown in a. Phosphorylation of dynamin in intact synaptosomes yields identical phosphopeptide maps to that shown for PKC (lane 5). Short arrows indicate positions of 21 and 19K PKC phosphopeptides, and long arrows those of the ~17K casein kinase II phosphopeptide. c, GTP hydrolysis by dynamin. Dynamin was stoichiometrically phosphorylated by PKC or casein kinase II or incubated in the absence of kinases (control) and reperfused to remove the kinases and ATP. GTPase activity was measured in all samples in quadruplicates, with the results shown for PKC phosphorylated and control dynamin derived from two independent experiments, and the CK-II results from a single experiment. Data for all conditions has been replicated in independent experiments at the 60 min time point.

METHODS. a, b, Purified dynamin (0.25 μg) was phosphorylated with the

various protein kinases (20 ng) in a reaction mixture (40 μl) containing 30 mM Tris-HCl pH 7.4, 1 mM MgCl₂, 1 mM EGTA, 40 μM ATP and 3.75 μCi [γ -³²P]ATP in the presence (where appropriate) of 10 μM cGMP or 200 μM free Ca²⁺ plus 10 mg l⁻¹ phosphatidylserine¹³. Protein kinases were purified as described in the following references: PKC¹³, cGMP-dependent protein kinase¹⁹, except that the kinase was further purified by an additional mono-Q step; cAMP-dependent protein kinase catalytic subunit²⁰, additionally followed by S-Sepharose chromatography. Casein kinase II was a gift of A. Tiganis and B. E. Kemp, Melbourne. Each enzyme was more than 95% pure as judged by Coomassie-stained gels. Phosphopeptide mapping was done as described¹⁰. c, Purified dynamin (100 μg) was phosphorylated as above in a 4 ml volume containing 1 μg of the respective kinases for 30 min at 30 °C. The control was incubated without additions. After the incubations, dynamin was reperfused on a 0.2 ml S-Sepharose column (Pharmacia), desalted on a 1 ml Sephadex G-25 column, and stored at -80 °C. GTPase assays were at 30 °C for the indicated times in a reaction (40 μl) containing 30 mM Tris-HCl pH 7.4, 2 mM Mg²⁺, 1 mM EGTA, 30 mM NaCl, 1 mM [γ -³²P]GTP (55 c.p.m. nmol⁻¹; DuPont), and 3 μg dynamin. Incubations were terminated by addition of 0.6 ml of 2% formic acid, 8% acetic acid. Released ³²P_i was detected as described²¹.

*vivo*¹⁰. V8 cleavage of purified 94 and 96K dynamin forms results in single phosphopeptides of 21 and 19K, respectively (data not shown), indicating that the PKC phosphopeptides include the sequences that differ in length between the 94 and 96K forms. Because this difference was shown above to localize to the C terminus of dynamin, the PKC phosphorylation site must be close to the C terminus, whereas the casein kinase II site must be further towards the N terminus.

Simultaneous phosphorylation of dynamin by casein kinase II and PKC does not result in increased phosphorylation. Instead, phosphopeptide mapping reveals that dynamin is only phosphorylated by casein kinase II but not by PKC under these conditions (Fig. 3b), indicating that casein kinase II phosphorylation inhibits PKC phosphorylation. Use of other PKC substrates demonstrates that casein kinase II does not have a direct effect on PKC activity (data not shown), suggesting that casein kinase II phosphorylation of dynamin at a distinct site may regulate PKC phosphorylation. cGMP-dependent protein kinase also inhibited dynamin phosphorylation by PKC. This kinase did not significantly phosphorylate dynamin but had a small direct inhibitory effect on PKC activity whose significance is currently unclear (Fig. 3a, b and data not shown).

To test the functional relevance of phosphorylation of dynamin, the intrinsic GTPase activity of dynamin was measured as a function of phosphorylation. For this purpose, purified dynamin was stoichiometrically phosphorylated by PKC and by casein kinase II and purified away from the kinases and ATP. Strikingly, PKC phosphorylation stimulated the GTPase activity of dynamin 12.1 ± 0.3-fold whereas casein kinase II phosphorylation had no effect (Fig. 3c). This suggests that the phosphorylation by PKC observed in synaptosomes directly regulates dynamin activity.

Previous studies have established that dynamin is a GTP-binding protein with two disparate properties. It is a microtubule-binding protein whose GTPase activity is stimulated by microtubules^{1,3}, suggesting a role in microtubule-based motility, and it is mutated in the *Drosophila* strain *shibire* that exhibits a general endocytosis defect^{4,5}, suggesting a role in a membrane-trafficking event that is not known to be microtubule-dependent. In the nerve terminal, synaptic vesicles endocytose rapidly after exocytosis to allow fast recycling, and it is here that the mutant phenotype of *shibire* becomes phenotypically manifest as paralysis^{6,9}. We have now shown that there are multiple forms of dynamin in brain that are major substrates for PKC in nerve terminals and are quantitatively dephosphorylated on stimulation. In addition to PKC, dynamin is phosphorylated by casein kinase II *in vitro* at a distinct site that has an inhibitory effect on PKC phosphorylation, suggesting a regulatory network of phosphorylation. The rapid dephosphorylation of dynamin in nerve terminals in parallel with synaptic vesicle exocytosis and recycling suggest a role for phosphorylation as a molecular switch for membrane traffic. The strong stimulation of dynamin GTPase activity by PKC phosphorylation, but not by casein kinase II phosphorylation, supports such a role as it demonstrates a regulation of dynamin GTPase in parallel with synaptic vesicle traffic. One possible model is that the downregulation of GTPase activity observed during stimulation-induced dephosphorylation in the nerve terminal may be instrumental in the rapid endocytosis of synaptic vesicles after neurotransmitter release. □

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Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity

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PEPTIDE hormones, neurotransmitters, and autacoids activate a family of seven-transmembrane-domain receptors¹. Each of these receptors specifically couples to one of several G proteins, G_s, G_i, G_o, and G_p, to activate a specific second messenger system². Cell surface receptors for prostanoids have been characterized pharmacologically³ and the complementary DNAs for thromboxane A₂ receptor^{4,5} and the EP3 subtype of the prostaglandin (PG)E receptor⁶ reveal that they belong to the seven-transmembrane-domain receptor family. The EP3 receptor mediates the diverse physiological actions of PGE₂ (ref. 3). Although most of them occur through coupling of the EP3 receptor to G_i and inhibition of adenylyl cyclase, the EP3-mediated contraction of uterine muscle can only occur by activation of another second messenger pathway⁷. In chromaffin cells, two different second messenger pathways are activated by PGE₂ binding to an apparently single EP3 receptor class⁸. Here we show that at least four isoforms of the EP3 receptor, which differ only at their C-terminal tails and are produced by alternative splicing, couple to different G proteins to activate different second messenger systems.

A complementary DNA library of bovine adrenal medulla was screened with a fragment of the mouse EP3 receptor cDNA as a probe under low-stringency conditions. Seven clones were isolated. Sequencing of these clones revealed four types of full-length clones which encoded amino acid sequences partially different from each other. Figure 1a shows that these clones, which have identical nucleotide and corresponding amino-acid sequences up to nucleotide 1,074 and amino acid residue 358, have different nucleotide sequences corresponding to C-terminal peptides. Because the common amino-acid sequence showed 80%

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homology with that of the mouse EP3 receptor, and hydrophobicity analysis revealed seven hydrophobic segments, we named the proteins encoded by the four clones EP3 A-D. Differences between these putative receptors begin ten amino acids after the seventh hydrophobic domain and extend to the carboxy terminus. The EP3A protein has the longest C-terminal tail, with 59 amino acids after the common sequence, followed by EP3D (29 amino acids), EP3B (27 amino acids) and EP3C

(4 amino acids). Among the cDNAs, EP3A, B and C contain the identical 3' sequence; cDNAs of EP3A and C have deletions of 308 and 14 nucleotides, respectively, compared to EP3B cDNA (Fig. 1b), but all four clones have the same 5' non-coding region. As shown in Fig. 2, the messenger RNA corresponding to each clone was detected by reverse transcription polymerase chain reaction (RT-PCR). In addition, genomic Southern blot analysis showed a single hybridization band with a probe for the

FIG. 1 a, Nucleotide and deduced amino-acid sequences of cDNAs of EP3 A-D. The common nucleotide sequence (nucleotide 1,1,074) is shown, followed by the sequences specific to each clone. Deduced amino acids are shown in single-letter code. The seven putative transmembrane domains are indicated by I to VII. b, The nucleotide sequences downstream of nucleotide 1,075 for EP3A (upper), EP3B (middle) and EP3C (lower). The sequences up to the termination codon for EP3A are shown. Coding regions are underlined. The three sequences are aligned and a deletion of a corresponding nucleotide is indicated by a dash. Note that consensus acceptor sequences (pyrimidine-rich regions followed by AG (bold letters)) are observed in the putative splicing sites. METHODS. cDNA of bovine adrenal medulla was prepared by the oligo(dT) priming method, size-selected (>1 kilobase) and inserted into the EcoRI site of the λ -ZAP II vector. Clones (5×10^5) were screened using the *PvuII*/*Bst*III fragment (428-bp) of the mouse EP3 receptor cDNA as a probe. Filters were washed twice at 60 °C in $2 \times$ SSC, 1% SDS for 30 min. Positive clones were isolated and excised *in vivo* into plasmids which served as templates for DNA sequencing. The numbers of clones isolated were: EP3A, 1; EP3B, 3; EP3C, 1; EP3D, 2. There was at least one full-length clone for each isoform. Hydrophobicity analysis was performed using the Kyte and Doolittle method²⁶.

a

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ATGAAGGCGACCCGAGACACCGGAGCGCTCTTCTGTACGCGCTTCAACCACTCCGACCCCGGCATCTGGCGCCGCGAGCGGCC :87
M K A T R D H A S A P F C T R F N H S D P G I W A A E R A :29
I
GTTGAGGCGCCAAACCACTCAGCTGCCCCCGGAACCCAGCGAGGACTGGCGCTCGGTCTGCTAGCCCTTTTCGATGACCATGATGATC :177
V E A P N N L T L P P E P S E D C G S V S V A F S M T M M I :59
II
ACCGGGTCTGGGCAACCGCTGGCCATAACGCTGTGTCTGAAGAGCTACAGCGCCGGAGGGGCAAGCCAGGAAGTCTGCTCTGCTG :267
T G F V G N A L A I T L V S K S Y R R R E G K R K K S F L L :89
TGCACTGGCTGGCTGGCTCACCACATGCTGGGCGAGCTGCTCACCAGCCGGTGGTGCATCTCTCTACCTGTCCACCAGCGCTGG :357
C I G W L A L T D M V G Q L L T S P V V I V L Y L S H Q R W :119
III
GAGCAGCTGACCCGCTGGGAGGTTATGCACCTTCTTGGGCTGACCTGACTGTCTTTGGGCTCTCTCTGCTCTTCATCTGCCAGCGCC :447
E Q L D P S G R L C T F F G L T M T V F G L S S L F I A S A :149
IV
ATGCGCGTCTGAGCGGGCTTGGCCACCCGGGCGCGCACTGTACTCAAGCCACATGAAGACAGCGCTCACCAGCGGCTGCTGCTAGGC :537
M A V E R A L A T R A P H W Y S S H M K T S V T R A V L L G :179
GTATGGCTGGCGGTGCTGCTCTGCGCCCTGTTGCGCGGTGCTGGGCTAGGTCTAGTATATCTATCTAGTGGCCGGGACTTGGTCTTCATT :627
V W L A V L A F A L L P V L G V G Q Y T I Q W P G T W C F I :209
V
AGCAGCGGGCTGGGCGCAACGGGCACTAATCCCGGCAAAATGGGGCAACGTTTCTTCTGCTCTGCTCTTGGCCTATCTGGGCTCTGCT :717
S T G P G G N G T N S R Q N W G N V F F A S A F A I L G L S :239
GCGCTGGTGGTCACTTCTGCTGCACTTGGCCACCATTAAGGCCCTAGTGTCCGCTGCAAGGCGCAAGGCCACGGCGTGCAGTCCAGC :807
A L V V T F A C N L A T I K A L V S R C R A K A T A S Q S S :269
VI
GCCAGTGGGCGGAATCAGACAGAGACGGCCATCCAGCTCATGGGATCATGCTGCTCTGCTCTGCTGCTGCTGCTGCTGCTGCTGATA :897
A Q W G R I T T E T A I Q L M G I M C V L S V C W S P L L I :269
VII
ATGATGTTGAATGATCTTCAATACACGCTCAGTTGAGCAGTCAAGACATACACAGAAATCAGGATGAGTGCACCTTCTTCTTAATA :987
M M L K M I F N H T Y E H C K T Y T E N Q D E C N F T C L I :329
GCTGTTCGCTGGCTTCACTGAACAGATCTTGGATCCCTGGGTTTATCTGTCTGCTAAGAAGATCTTCTTCAAAAGTTTTCGCG :1074
A V R L A S L N Q I L D P W V Y L L L R K I L L Q K F C Q :358

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EP3A

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CTTTTGAAAGGACACTCTTATGACTAGACACGGAGGGTGGGACAGAGAACAAGACAAGAGATGAAGGAAACCTATATATTTCCAAAC :1164
L L K G H S Y G L D T E G G T E N K D K E M K E N L Y I S N :388
TTGTCAAGATCTTCACTCTTTTAGGCCATTTACAGAGAAGGAGAGGAAGGGCCACATTTATCTTATACACTTGGAGATCAGTAG :1254
L S R F F I L L G H F T E A R R R G R G H I Y L H T L E H Q * :417

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EP3B

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GCCTCACCCCGCAGCATGTGGGATCTAGTTCCTCCGACACGGGATCGAACCCGGTCCCTGCTATGGAAGCAGGAGCTTTAA :1158
A S P R S M W D P S S P T R D R T R V P C I G S T E S * :385

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EP3C

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CATGTGGGATCTTAG :1089
H V G S * :362

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EP3D

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GTAGCAATGCTGTCTCCAGCTACTTTAATGATGGACCTAAAGTCCGACTATCTACTATCCAAATGAATAACACAGACAGGAGCATGA :1164
V A N A V S S Y F N D G P K V P T I S L S N E I T Q T G A * :387

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b

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A -----
B GCTTACCCCGCAGCATGTGGGATCTAGTTCCTCCGACACGGGATCGAACCCGGTCCCTGCTATGGAAGCAGGAGCTTTAATCTACTG :
C -----CATGTGGGATCTTAGTTCCTCCGACACGGGATCGAACCCGGTCCCTGCTATGGAAGCAGGAGCTTTAATCTACTG

A -----
B GACACACGGGAGTCCCTGTCTAGTTTCTAGATAGAAATGTTGGCTTTTAAATTAAGTGAATCAGATATCTAGCCAAAGCTTGG :
C GACACACGGGAGTCCCTGTCTAGTTTCTAGATAGAAATGTTGGCTTTTAAATTAAGTGAATCAGATATCTAGCCAAAGCTTGG

A -----
B CCAATCAGCAGAAATTAATATGAAAAATAGTTTCTTCTATGTTTCTCAATTTCTGTAGAACCAGAACTGAATGTTTAGCAGGAGAAAA :
C CCAATCAGCAGAAATTAATATGAAAAATAGTTTCTTCTATGTTTCTCAATTTCTGTAGAACCAGAACTGAATGTTTAGCAGGAGAAAA

A -----CTTTTGAAAGGACACTCTTATGACTAGACACGGAGGGTGGGACAGAGAGACA
B TCGGTATGACTTACTGATGACTTCAAGCTTTTCAAGCTTTTGAAGGACACTCTTATGACTAGACACGGAGGGTGGGACAGAGAGACA
C TCGGTATGACTTACTGATGACTTCAAGCTTTTCAAGCTTTTGAAGGACACTCTTATGACTAGACACGGAGGGTGGGACAGAGAGACA

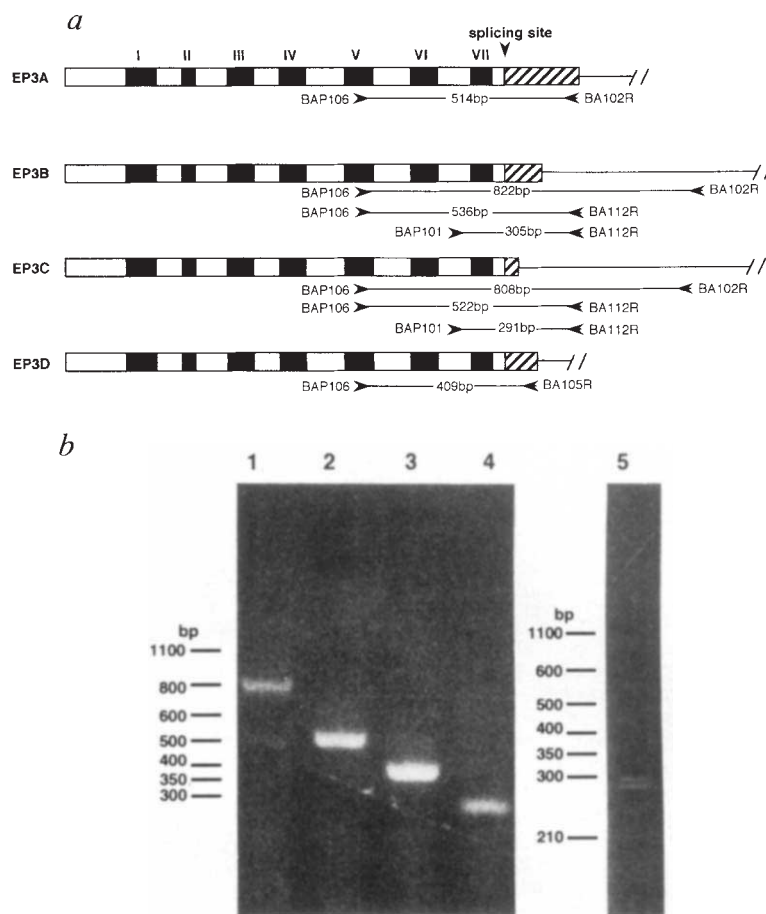
A AAGACAAAGAGATGAAGGAAACCTATATATTTCCAACTTGTCAAGATCTTCTATCTTTTAGGCCATTTTCACAGAACGAGGAGAGAA
B AAGACAAAGAGATGAAGGAAACCTATATATTTCCAACTTGTCAAGATCTTCTATCTTTTAGGCCATTTTCACAGAACGAGGAGAGAA
C AAGACAAAGAGATGAAGGAAACCTATATATTTCCAACTTGTCAAGATCTTCTATCTTTTAGGCCATTTTCACAGAACGAGGAGAGAA

A GGGGCCACATTTTATCTTATACACTTGGAGCATCAGTAG .....
B GGGGGCAGATTTTATCTTATACACTTGGAGCATCAGTAG .....
C GGGGGCAGATTTTATCTTATACACTTGGAGCATCAGTAG .....

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FIG. 2 RT-PCR of the four isoforms of EP3 mRNA. *a*, Schematic presentation of PCR primers, corresponding positions in each cDNA and expected sizes of PCR products. Transmembrane domains are shown by closed boxes and different C-terminal tails by hatched boxes. *b*, Gel electrophoresis of amplified products. Lanes 1 to 5 correspond to PCR analyses with a primer pair of BAP106 (primer corresponding to the fifth transmembrane domain) and BA102R (primer specific for the C terminus of EP3 A, B and C) in lane 1; BAP106 and BA112R (primer specific for the C terminus of EP3 B and C) in lane 2; BAP106 and BA105R (primer specific for the C terminus of EP3D), in lane 3; BAP101 (primer corresponding to the third intracellular loop) and BA112R primer in lanes 4 and 5.

METHODS. Total RNA of bovine adrenal medulla was obtained by the AGPC method⁵. By using MMLV reverse transcriptase (BRL), first-strand cDNA was prepared as described⁵. Sequences of the PCR primers were BAP101, TCAGGATGAGTGCAACTT; BAP106, CACCTTCGCCTGCAACTT; BA102R, CAAGTGATGAAGATAAATGTGG; BA112R, CTGGTGATTGGCAAGCTTTTGG; BA105R, TGGATAGTGAGATAGTCGGCACT. PCR was performed on a Zymoreactor (ATTO) with 28 cycles of 0.6 min of denaturation at 94 °C, 0.6 min of annealing at 57 °C and 1.0 min of extension at 72 °C. Samples were electrophoresed in 1.5% agarose gel (lane 1 to 4) or in 6% polyacrylamide gel (lane 5). Control experiments in the absence of reverse transcriptase produced no bands.



common nucleotide sequence (data not shown). These results taken together indicate that the variation in C-terminal peptides could be produced by alternative splicing of mRNA from the single gene encoding the EP3-receptor. Sequences around the putative junctions fit well with the proposed consensus sequences for RNA splicing⁹ (Fig. 1b).

To confirm the receptor subtype encoded by these clones, we examined the ligand specificities of each EP3 receptor expressed on CHO cells. Membrane fractions of the cells expressing EP3 A-D showed specific [³H]PGE₂ binding, with *K_d* values of 2.5, 6.5, 6.2 and 2.3 nM, respectively. These bindings were displaced efficiently by a specific EP3 agonist, MB-28767, as well as by PGE₂ and PGE₁. Displacement by other PGs and the EP1 and EP2 ligands, such as iloprost, PGF_{2α}, PGD₂, SC-19220 and butaprost, required concentrations that were at least two orders of magnitude higher. The rank order of potency for these compounds in displacing labelled PGE₂ was identical for the four receptors (data not shown). These results indicate that all of the four cDNAs encode the EP3 subtype of the PGE receptor and that the divergence in the C-terminal tails do not change the ligand specificities. These binding properties are similar to those of the PGE receptor in chromaffin cells and the small differences in the *K_d* values for the four isoforms are consistent with the single class of [³H]PGE₂ binding observed in these cells^{8,10}.

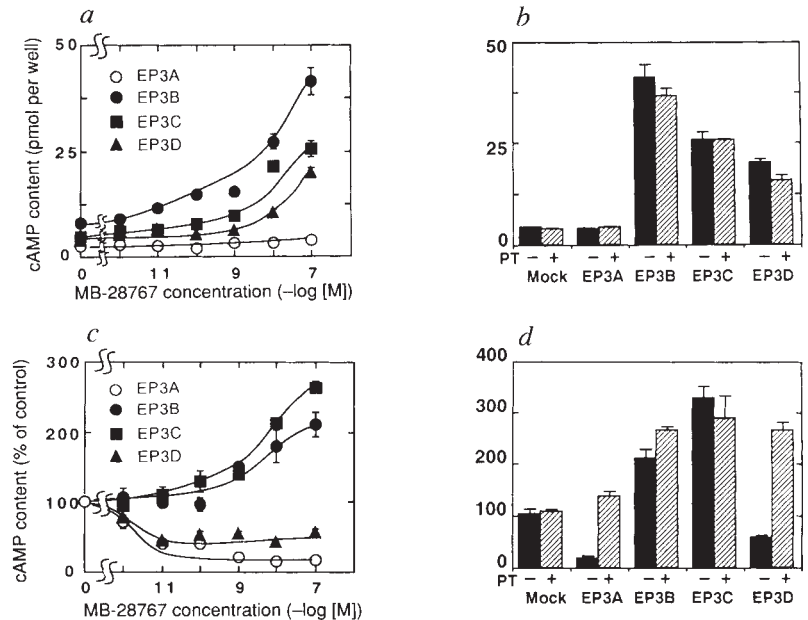
Because a previous study⁸ showed that the PGE receptor in chromaffin cells couples to multiple second messenger systems, we examined the signal transduction mediated by the four isoforms. Figure 3a shows that MB-28767 increased the level of cyclic AMP in a concentration-dependent fashion between 10 pM and 100 nM in cells expressing the EP3 B-D isoforms, but not in those cells expressing the A isoform. Responses in the B-D isoforms were insensitive to pertussis toxin treatment (Fig.

3b). The compound MB-28767 also stimulated cAMP generation in membranes of cells expressing these three isoforms in a GTP-dependent manner, and this response was inhibited by a specific anti-G_{sa} antibody (data not shown), suggesting that these isoforms stimulate adenylyl cyclase by coupling to G_s and not by liberating βγ subunits from G_i protein, as postulated for the α₂-adrenergic receptor¹¹. We next determined whether these isoforms could inhibit adenylyl cyclase. The inhibition by MB-28767 of forskolin-induced cAMP generation was observed in cells expressing the EP3 A and D isoforms (EC₅₀, 10 pM; Fig. 3c), and was sensitive to pertussis toxin treatment (Fig. 3d). When the cells were treated with pertussis toxin, MB-28767 potentiated forskolin-induced cAMP generation in the EP3D-expressing cells to a level comparable to that from EP3B- and EP3C-expressing cells, whereas there was little potentiation in EP3A-expressing cells.

We also tested for agonist-induced phosphatidylinositol (PtdIns) turnover in each CHO cell line. As shown in Fig. 4a, MB-28767 at 1 μM stimulated significant production of inositol trisphosphate (InsP₃) in the EP3A- and EP3D-expressing cells, while not in cells expressing EP3B and C. Pertussis toxin partially reduced PtdIns turnover by the EP3D isoform and abolished it in EP3A-expressing cells. These results indicate that most, if not all, of the PtdIns turnover by EP3A was evoked by coupling of this receptor to G_i/G_o, whereas EP3D couples to a G protein of the G_p class, to elicit most of this response. The agonist-induced increase in intracellular free calcium ion concentration ([Ca²⁺]_i) was consistently observed in Fura-2-loaded CHO cells expressing the EP3 A and D isoforms (Fig. 4b, c); pertussis toxin treatment abolished the response in EP3A cells but not that in the EP3D cells (Fig. 4d, e). There was no increase in [Ca²⁺]_i in EP3B- and EP3C-expressing cells. These results taken together indicate that EP3A couples to G_i/G_o protein,

FIG. 3 Effects of MB-28767, an EP3 agonist, on cAMP levels in CHO cells. *a*, MB-28767-induced cAMP accumulation. Cells expressing each isoform were incubated with various concentrations of MB-28767 and the cAMP level measured. *b*, Effect of pertussis toxin on the agonist-induced accumulation of cAMP. Cells were cultured with or without pertussis toxin for 12 h and then incubated with 100 nM MB-28767. *c*, MB-28767-induced inhibition of forskolin-induced cAMP accumulation. Cells were incubated with 3 μ M forskolin and various concentrations of MB-28767 and cAMP amounts were measured. *d*, Effect of pertussis toxin on the agonist-induced inhibition of forskolin-induced cAMP accumulation. Cells were cultured with or without pertussis toxin for 12 h and then incubated with 3 μ M forskolin and 100 nM MB-28767.

METHODS. cDNAs encoding each isoform were inserted into the mammalian expression vector, pdkCR-dhfr and introduced into the CHO-dhfr⁻ cells²⁷ by lipofection²⁸. Stable transformants were cloned by selection in the α -modification of minimal essential medium containing 10% dialysed fetal calf serum without nucleotides. $B_{\max}$ values in cells expressing EP3A–D were 1.9, 9.8, 2.0, and 7.8 pmol mg⁻¹ protein, respectively. Cells were cultured in 24-well plates in the medium to ~80% confluency (~5 $\times 10^5$ /well) and treated with or without 20 ng ml⁻¹ pertussis toxin for 12 h. They were then rinsed with HEPES-buffered saline and preincubated with 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37 °C. MB-28767 was added with or without 3 μ M forskolin. After incubation for 10 min at 37 °C, the reaction was terminated by adding ice-cold trichloroacetic acid. cAMP content was measured by radioimmunoassay (Amersham). Adenylyl cyclase stimulation did not depend on the level of the receptor expression, because stimulation of the enzyme was also observed in a cell line expressing EP3B with a $B_{\max}$ value of 1.5 pmol mg⁻¹. Pertussis toxin (PT) treatment was confirmed by [³²P]ADP-ribosylation as



described²⁹. All values are expressed as means $\pm$ s.e.m. ($n = 3$). Control cAMP level in *c* were 49.9 ± 0.9 , 47.4 ± 3.4 , 28.4 ± 1.2 , 45.0 ± 0.2 pmol per well for cells expressing EP3 A–D, respectively. cAMP levels (pmol per well) in control cells without MB-28767 in *d* were 62.0 ± 2.7 (PT⁻) and 43.2 ± 2.9 (PT⁺) in mock-treated cells, 48.2 ± 3.4 (PT⁻) and 43.8 ± 1.8 (PT⁺) in EP3A-expressing cells, 48.6 ± 3.9 (PT⁻) and 69.5 ± 4.6 (PT⁺) in EP3B-expressing cells, 12.5 ± 0.9 (PT⁻) and 61.5 ± 4.8 (PT⁺) in EP3C-expressing cells and 26.4 ± 1.1 (PT⁻) and 28.0 ± 1.2 (PT⁺) in EP3D expressing cells, respectively.

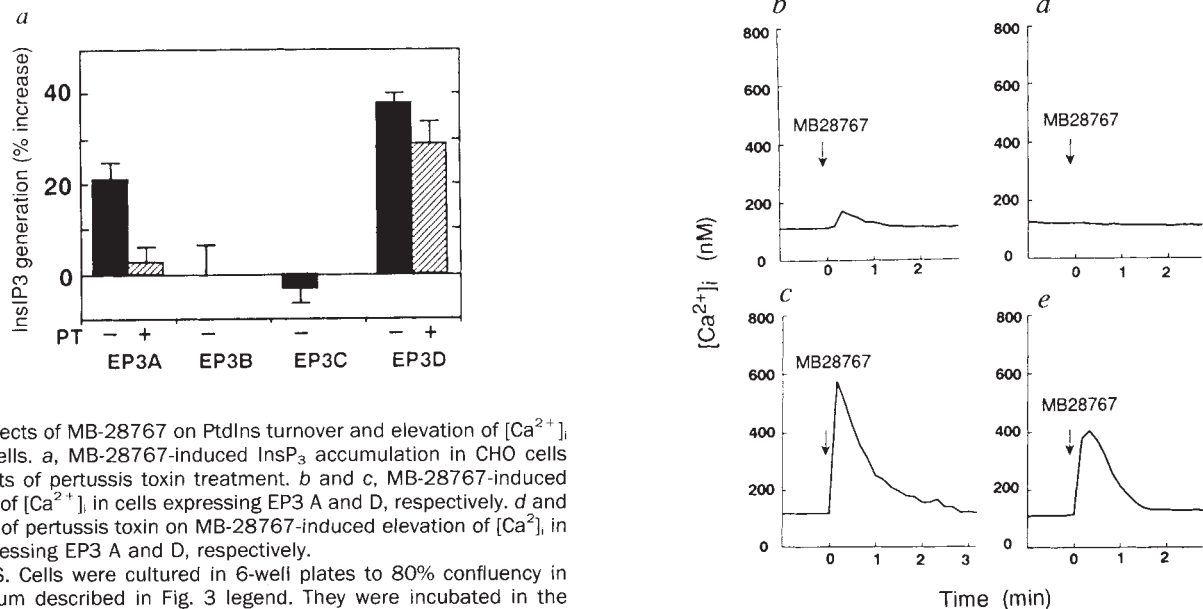


FIG. 4 Effects of MB-28767 on PtdIns turnover and elevation of $[Ca^{2+}]_i$ in CHO cells. *a*, MB-28767-induced InsP₃ accumulation in CHO cells and effects of pertussis toxin treatment. *b* and *c*, MB-28767-induced elevation of $[Ca^{2+}]_i$ in cells expressing EP3 A and D, respectively. *d* and *e*, Effects of pertussis toxin on MB-28767-induced elevation of $[Ca^{2+}]_i$ in cells expressing EP3 A and D, respectively.

METHODS. Cells were cultured in 6-well plates to 80% confluency in the medium described in Fig. 3 legend. They were incubated in the presence or absence of 20 ng ml⁻¹ pertussis toxin for 12 h in inositol-free RPMI 1640 medium with 10% dialysed fetal calf serum containing 1 μ Ci ml⁻¹ myo-[2-³H]inositol. After washing with HEPES-buffered saline and preincubating with 10 mM LiCl in 1.8 ml solution at 37 °C for 5 min, MB-28767 in 200 μ l was added to a final concentration of 1 μ M. After 30 s, medium was removed and the reaction terminated by adding 1 ml ice-cold 5% trichloroacetic acid. InsP₃ was measured as described⁹ and expressed as the per cent increase from control (mean $\pm$ s.e.m., $n = 6$). The radioactivity (d.p.m. per dish) in the cells without agonist were

3,547 $\pm$ 59 (PT⁻) and 1,939 $\pm$ 28 (PT⁺) in EP3A expressing cells, 2,136 $\pm$ 211 (PT⁻) in EP3B-expressing cells, 1,987 $\pm$ 35 (PT⁻) in EP3C-expressing cells, and 3,143 $\pm$ 53 (PT⁻) and 1,349 $\pm$ 48 (PT⁺) in EP3D-expressing cells, respectively. InsP₃ generation by the EP3D isoform began 10 s after addition of the MB-28767 compound and peaked (220% of control) at 60 s. EC₅₀ for InsP₃ generation was ~10 nM. Elevation in $[Ca^{2+}]_i$ on application of 1 μ M MB-28767 was recorded as described³⁰.

whereas EP3 B and C couple to G_s , and EP3D to G_i , G_s and G_p . The same results were obtained in other CHO clones transfected with each of the four cDNAs.

Our results show that the rhodopsin-type receptors, which have identical intracellular loops and differ only in their C-terminal tails, have different specificities for coupling to G proteins. The domains of the rhodopsin-type receptor that interact with G protein and determine this specificity have been studied using chimaeric receptors¹² or receptors carrying various deletion or point mutations¹²⁻¹⁴, and by examining the effects of partial receptor peptides on G proteins¹⁵⁻¹⁷. These studies suggested the importance of a short sequence of amino acids in the second and third intracellular loops in the β_2 and α_2 adrenergic receptors and the M4 muscarinic receptor. On the other hand, the C-terminal sequence is believed to contribute to coupling efficiency

between receptor and G protein^{12,18,19}, and there has been no evidence that it is involved in the selection of G proteins. We have thus revealed a novel type of receptor-G-protein recognition in that the C-terminal tail, if not directly interacting with the G protein, determines the specificity of coupling. The isoforms analysed here are probably produced by alternative splicing of mRNA, which generates multiple protein isoforms from a single gene. Since the first description of two isoforms for the D₂ dopaminergic receptor^{20,22}, production of multiple isoforms by alternative splicing has been reported in the rhodopsin-type receptor family²³⁻²⁵. However, none of these show any differences in their signalling pathways. The EP3 isoforms activate different second messenger cascades without changing ligand-binding specificity, and are therefore an example of alternative splicing contributing to physiologically different receptor activity³. □

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Differential signal transduction by five splice variants of the PACAP receptor

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THE two forms of pituitary adenylyl cyclase-activating polypeptide (PACAP-27 and -38) are neuropeptides of the secretin/glucagon/vasoactive intestinal polypeptide/growth-hormone-releasing hormone family and regulate hormone release from the pituitary and adrenal gland¹⁻³. They may also be involved in spermatogenesis⁴, and PACAP-38 potently stimulates neurite outgrowth and survival of cultured rat sympathetic neuroblast^{5,6} and promotes neurite outgrowth of PC-12 cells⁷. The PACAP type-I receptor (found in hypothalamus, brain stem, pituitary, adrenal gland and testes), specific for PACAP, is positively coupled to adenylyl cyclase and

phospholipase C. The recently cloned type II receptor does not discriminate between PACAP and vasoactive intestinal polypeptide and is coupled to only adenylyl cyclase⁸. Here we have used a new expression cloning strategy, based on the induction of a reporter gene by cyclic AMP, to isolate a complementary DNA encoding the type-I PACAP receptor. On transfection of this cDNA, both PACAP-27 and -38 stimulate adenylyl cyclase with similar EC₅₀ values (50% effective concentration, 0.1-0.4 nM), whereas only PACAP-38 stimulates phospholipase C with high potency (EC₅₀ = 15 nM). Four other splice variants were isolated with insertions at the C-terminal end of the third intracellular loop. Expression of these cDNAs revealed altered patterns of adenylyl cyclase and phospholipase C stimulation, suggesting a novel mechanism for fine tuning of signal transduction.

A novel expression cloning method was used to screen simultaneously for different receptors positively coupled to adenylyl cyclase. This method is based on transcriptional induction of a cAMP-responsive luciferase reporter gene by stimulation of adenylyl cyclase through activated target receptors (Fig. 1). Pools of clones from a newborn rat brain colliculi cDNA library were amplified and the DNA was cotransfected with a reporter construct into LLC PK1 cells (Fig. 1), which do not express endogenous PACAP and vasoactive intestinal polypeptide (VIP) receptors. Of 150 pools tested, one consistently stimulated luciferase activity in the presence of PACAP-27 (100 nM) but not VIP. A functional clone (139-453-40), containing a 1.9-kilobase (kb) insert, was subsequently isolated by successive subdivisions. This cDNA contains no in-frame stop codons and corresponds to a truncated form of the PACAP receptor, lacking the 35 C-terminal amino-acid residues. A second clone (140-1), extending from the putative third transmembrane domain to 1.2 kb downstream of the stop codon, was isolated by cross-hybridization.

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The sequences of the two cDNAs are identical in their 644-nucleotide overlapping region with the notable exception of 84 additional nucleotides encoding 28 amino acids in clone 140-1. The composite cDNA (Fig. 2a) encodes a protein with seven putative membrane-spanning domains. The insertion occurs at the C-terminal end of the third intracellular loop, a domain thought to be crucial for interaction with G proteins in many receptors⁹.

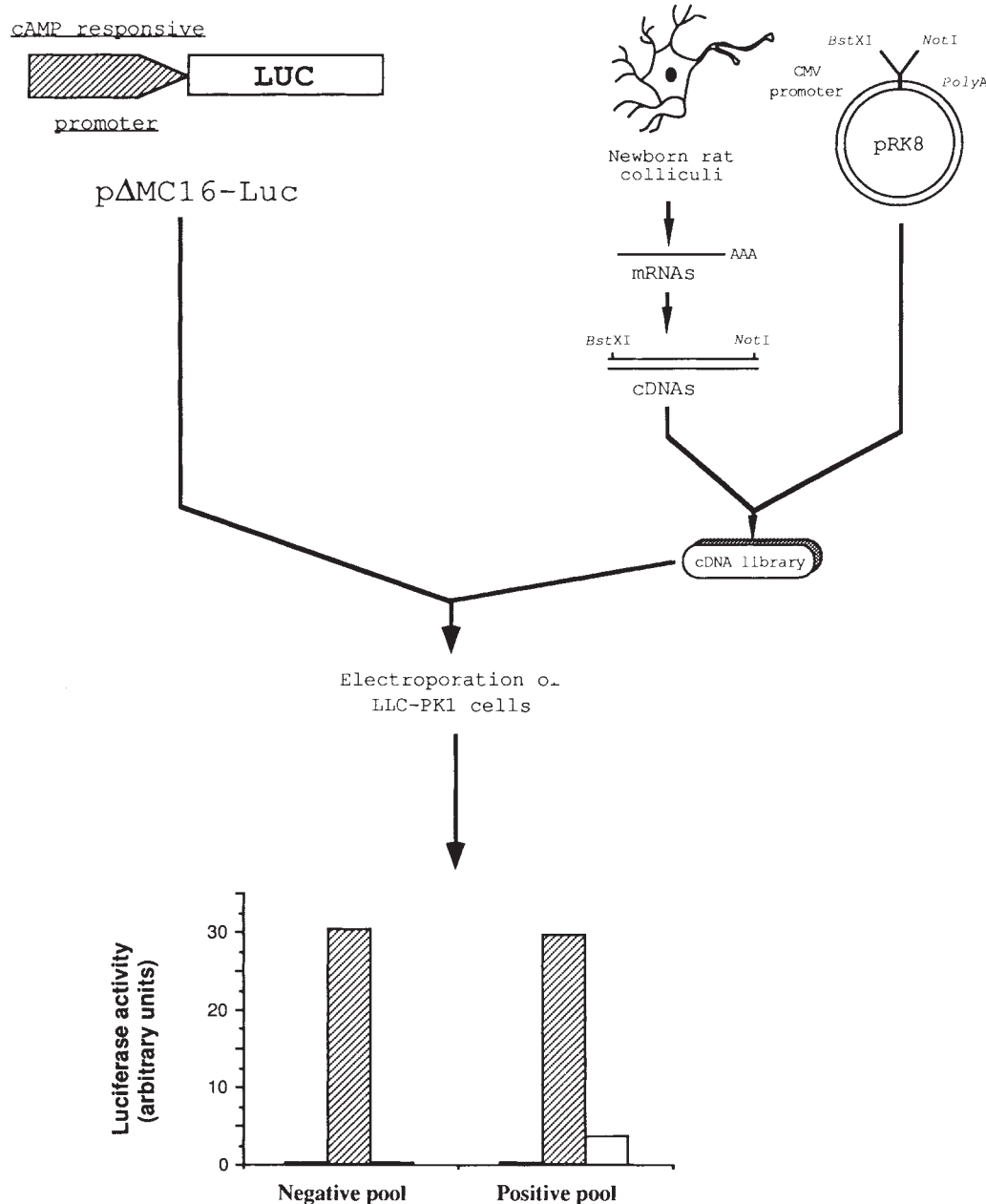
The amino-acid sequence of the PACAP receptor is markedly homologous to that of the rat VIP (54%), secretin (50%), glucagon (36%) and growth-hormone-releasing hormone (GHRH; 40%) receptors^{8,10,14}, but not to that of other G-protein-coupled receptors. To isolate additional variants, we used the polymerase chain reaction (PCR) with primers located in the 5' and 3'-untranslated regions on the cDNAs used to construct the library. We isolated five full-length cDNAs whose sequences are identical to that of the originally isolated clones but divergent from each other by the absence or presence of either one or two 84-bp cassettes, designated 'hip' and 'hop' (Fig. 2b). The splice variant of the recently cloned GHRH receptor contains an insertion at

exactly the same position as the PACAP receptor¹². These isoforms suggest an alternative splicing mechanism of the PACAP receptor gene. Therefore, we screened a rat genomic library with a PCR fragment containing the coding sequence of the cassettes and isolated 22 clones out of 2×10^6 phages. Partial sequencing of clone g5-1, comprising both cassettes as well as up- and downstream regions, revealed exon-intron boundaries in excellent agreement with consensus exon-intron junction sequences¹⁵ (Fig. 2c). The alternative use of two contiguous consensus splice acceptor sites at the 5' end of the hop cassette probably generates PACAP-R hop1 and -2 (Fig. 2c). Other examples of alternatively spliced G-protein-coupled receptor include the D₂ dopaminergic^{16,18} and the GHRH receptor^{12,13}, for which no clear functional differences emerged, whereas for the alternatively spliced metabotropic glutamate receptor¹⁹ (mGluR1) modified signal transduction properties have been reported²⁰.

All five clones were transiently expressed in LLC PK1 cells and the production of both cAMP and inositol phosphates was quantified. Dose-response curves obtained for the shortest variant (PACAP-R) display similar potency for PACAP-38 and -27

FIG. 1 Schematic representation of the expression cloning method. Histogram: ■, basal; ▨, 100 nM arginine vasopressin; □, 100 nM PACAP-27.

METHODS. Poly(A)⁺ RNA was prepared from newborn rat colliculi. Reverse transcription was performed with a random primer-Not I adapter (5'-ATG-TCTCGAGGCCTTTGCGGCCGCT-ATANNNNNNN-3'). After second-strand synthesis, *Bst*XI adaptors (in Vitrogen) were added. The cDNAs were digested with *Not*I, size-selected on a polyacrylamide gel and cloned in the *Bst*XI/*Not*I sites of pRK 8, a modified pRK5 vector²⁷. After electrotransformation of DH5 α , pools of about 2,000 clones were amplified and DNA (10 μ g) was cotransfected into porcine renal epithelial LLC PK1 cells with the luciferase reporter plasmid p Δ M-C16-LUC (1 μ g) and plasmid pCH110 (1 μ g), containing a SV40-promoter-driven β -galactosidase gene. The construct p Δ M-C16-LUC contains a modified mouse mammary tumour virus promoter in which the glucocorticoid-responsive region was replaced by 16 copies of a cAMP-responsive element derived from the promoter of the corticotropin-releasing hormone gene²⁸. Aliquots of the electroporated cells were plated in 12-well clusters and hormones were added 24 h later for 4 h. The ratio of luciferase to β -galactosidase activities of the cell lysates in the presence or absence of hormone was used as an index for the expression of the respective receptor in the transfected pool. Endogenous V₂ vasopressin receptors were used as a positive control in each transfection experiment.



a

-90 ccggagaccagcagcagtgagacagtgaggcgggtgactgaatctccaagctctggaacaatagccaga

-20 gatagtgctggggaagcaccATG GCC AGA GTC CTG CAG CTC TCC CTG ACT GCT CTC CTG CCT

45 GTG GCT ATT GCT ATG CAC TCT GAC TGC ATC TTC AAG AAG GAG CAA GCC ATG TGC CTG GAG

165 ATG TGG GAC AAT ATC ACA TGT TGG AAG CCA GCT CAA GGT GAG ATG GTC CTT GTA AGC

225 TGC CCT GAG GTC TTC CGG ATC TTC AAC CCG CAG CAA GTC TGG ATG ACA GAA ACC ATA GGA

285 GAT TCT GGT TTT GCC GAT AGT AAT TCC TTG GAG ATC ACA GAC ATG GGG GTC GTG GGC CGG

345 AAC TGC ACA GAG GAC GGC TGG TCG GAG CCC TTC CCC CAC TAC TTC GAT GCT TGT GGG TTT

405 GAT GAT TAT GAG CCT GAG TCT GGA GAT CAG GAT TAT TAC TAC CTG TCG GTG AAG GCT CTC

465 TAC ACA GTC GGC TAC AGC ACT TCC CTC GCC ACT CTC ACT GTC ATC TTC TGC

525 CGC TTC CGG AAG CTG CAT TGC ACT CGC AAC TTC ATC CAC ATG AAC CTG TTT GTA TCC TTC

585 ATG CTG AGG GCT ATC TCC GTC TTC ATC AAG GAC TGG ATC TTG TAC GCC GAG CAG GAC AGC

645 AGT CAC TGC TTC GTT TCC ACC GTG GAG TGC AAA GCT GTC ATG GTT TTC TAC CAC TAC TGC

705 GTG GTG TCC AAC TAC TTC TGG CTG TTC ATT GAA GGC CTG TAC CTC TTT ACA CTG CTG GTG

765 GAG ACC TTC TTC CCT GAG AGG AGA TAT TTC TAC TGG TAC ACC ATC ATC GGC TGG GGC APT

825 CCT ACT GTG TGT GTA ACA GTG TGG GCT GTG CTG AGG CTC TAT TTT GAT GAT GCA GGA TGC

885 TGG GAT ATG AAT GAC AGC ACA GCT GTG TGG TGG GTG ATC AAA GGC CCC GTG GTT GGC TCT

945 ATA ATG GTT AAC TTT GTG CTT TTC ATC GGC ATC ATC ATC ATC CTT GTA CAG AAG CTG CAG

1005 TCC CCA GAC ATG GGA GGC AAC GAG TCC AGC ATC TTA CGG CTG GCC CGC TCC ACC CTA

1065 CTG CTC ATC CCA CTC TTC GGA ATC CAC TAC ACA GTA TTC GCC TTC TCT CCA GAG AAC CTG

1125 AGC AAG AGG GAA AGA CTT GTG TTT GAG CTT GGG CTG GGC TCC TTC CAG GGC TTT GTG GTG

1185 GCT GTA CTC TAC TGC TTC CTG AAT GGG GAG GTA CAG GCA GAG ATT AAG AGG AAA TGG AGG

1245 AGC TGG AAG GTG AAC CGT TAC TTT ACT ATG GAC TAC AAG CAC CGG CAG CCG TCC CTC GCC

1305 AGC ACT GGA GTA AAT GGG GGA ACC CAG CTG TCC ATC CTG AGC AAG AGC AGC TCC CAG CTC

1365 CGC ATG TCC AGC CTC CCG GCC CAG AAC TTG GCC ACC TGA ggcctgtctccctccctctctgacag

1452 cctcccaagggaagagagagataggataggctgatatt

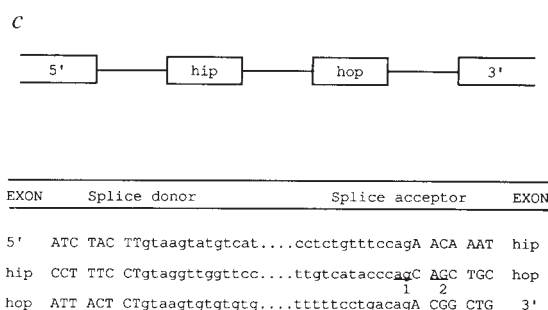


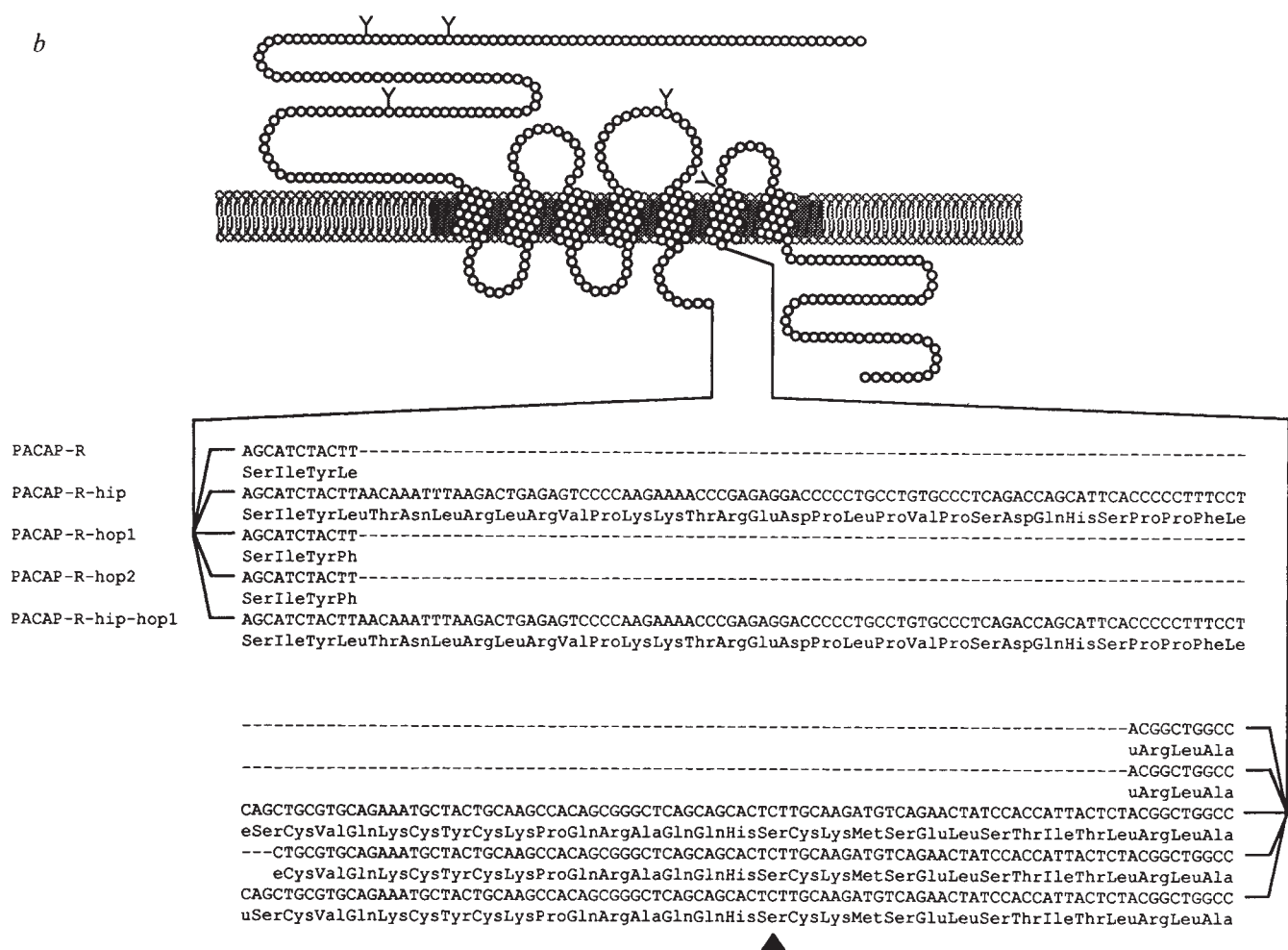
FIG. 2 Nucleotide and predicted amino-acid sequences of five splice variants of the rat PACAP receptor and nucleotide sequences of the exon-intron boundaries of the PACAP-R gene. **a**, Nucleotide sequence of the composite rat cDNA for the PACAP receptor derived from clones 139-453-40 and 140-1. The ATG of GCACCATGG is assigned as initiation codon on the basis of its close match to the CC(A/G)CATGG Kozak consensus sequence for favoured initiation of translation²⁹ and the presence of an in-frame TAG codon 15 nucleotides upstream. The amino acids predicted to form transmembrane domains by the Kyte and Doolittle³⁰ hydrophobicity analysis are underlined. There is no consensus sequence for phosphorylation by protein kinases. Five potential Asn-X-Ser/Thr glycosylation sites are indicated (*). **b**, Predicted topology of the PACAP-R receptor splice variant and sequences of four other splice variants. The 5' cassette hip encodes 84 bp inserted at position 1,043 of the short splice variant. The 3' cassette hop consists of 84 bp inserted at the same position. The cassette hip can also be included together with hop1 to give rise to hip-hop1. One consensus sequence for phosphorylation by protein kinase C is marked in the hop cassette (arrowhead). **c**, Diagram of the PACAP receptor gene showing the alternative splicing sites. Two contiguous splice acceptor sites in the 5' end of the hop cassette are underlined and numbered 1 and 2. **METHODS.** **a, b**, PCR was performed on the cDNA used in library construction. Primers NC1E (5'-AGAGAATTCCTGGAGACCAGCAGCGAG-TGGACAGT-3') and NC3X (5'-AGACTCTAGAAATATCAGCCTATCCCTATCTC-TCTCTTCTT-3') are located in the 5'- and 3'-untranslated regions of the PACAP-R receptor and contain restriction sites for *EcoRI* and *XbaI*, respectively. PCR products were restricted with *EcoRI*/*XbaI*, subcloned into pRK5, sorted by C-tracking and at least two clones were sequenced entirely on both strands. **c**, A rat genomic library (CLONTECH Laboratories) constructed in EMBL-3 phage from DNA partially digested with *Sau3A* was screened (2×10^6 phages) using a 460-bp PCR fragment of the PACAP receptor cDNA containing the cassettes hip and hop to generate a random-primed probe. Positive clones ($n=22$) were further sorted by hybridization with oligonucleotides specific for each cassette. Restriction fragments from clone g5-1 hybridizing with oligonucleotides specific for the cassettes and the upstream and downstream regions were subcloned in pBSbluescript and sequenced using T3, T7 and internal primers.

in adenylyl cyclase stimulation (0.1–0.4 nM) (Fig. 3a), whereas PACAP-38 displays higher potency than PACAP-27 in stimulating phospholipase C (15 nM versus $>1 \mu\text{M}$ respectively) (Fig. 3b). Confirming the specificity of the receptor for PACAP, VIP is at least 1,000-fold less potent in stimulating adenylyl cyclase and not active at all in phospholipase C stimulation. The related peptides glucagon, peptide histidine-methionine (PHM) and secretin were ineffective for both adenylyl cyclase and phospholipase C stimulation. These results are consistent with the pharmacology of the PACAP receptors described for PC-12 cells⁷ and for primary cultures of embryonic colliculi neurons (A. Demuis, personal communication). Similar results were obtained for PACAP-R-hop1 or -hop2 (Table 1). Interestingly, the hop cassette encodes a consensus motif for phosphorylation by protein kinase C (Fig. 2b), possibly contributing to the fine-tuned regulation of receptor function. In contrast, the presence of the hip cassette impairs adenylyl cyclase stimulation and abolishes phospholipase C stimulation. Finally, PACAP-R-hip-hop1 dis-

plays an intermediate phenotype with slightly reduced potency for both transduction pathways and impairment of efficiency for phospholipase C stimulation.

We extended these observations on differential coupling of receptor variants to phospholipase C by injection of the corresponding RNAs into *Xenopus* oocytes and recording of calcium-activated chloride currents (Fig. 3c). On application of PACAP-38, PACAP-R (Fig. 3c) as well as PACAP-R-hop1 and -hop2 (data not shown) elicit fast and large responses. In contrast, PACAP-R-hip-hop1 induces oscillating and small currents, and PACAP-R-hip elicits no response at all in this system. Similar results were recently reported for splice variants of the metabotropic glutamate receptor mGluR1 (ref. 20) and for the m2 and m3 subtypes of the muscarinic acetylcholine receptor. These results suggest coupling to different G proteins, as demonstrated for the m2 and m3 subtypes^{21,22}.

In situ hybridization experiments on adult rat brain and selected peripheral tissues with an oligonucleotide not discrimi-



nating between the different splice variants indicate a distribution of PACAP receptor messenger RNA compatible with autoradiographic and binding data^{4,23,25}. Briefly, prominent expression was noted in olfactory bulb, thalamus, hypothalamus, colliculi, dentate gyrus, occipital cortex, cerebellum, intermediate and anterior pituitary and adrenal medulla (Fig. 4a-e). To study the expression of the different PACAP receptor isoforms we performed reverse transcription (RT)-PCR with primers adjacent to the site of insertion of the cassettes on different

regions of the adult brain and peripheral tissues. Amplification produced two major bands (Fig. 4f) of the expected size, corresponding to the short form (PACAP-R) and to a form with one cassette which was identified as PACAP-R hop by hybridization with a cassette-specific oligonucleotide (data not shown). Interestingly, the ratio between the two major PCR products varies between tissues. In adult cerebral cortex, hippocampus, thalamus plus hypothalamus, brain stem, cerebellum and pituitary, PACAP-R is the most abundant form, whereas PACAP-R hop predominates in the PCR products of the testes, olfactory bulb and adrenal gland. Therefore, the splicing of the PACAP receptor gene seems to be differentially regulated in adult tissues. As the hip cassette was not detected in adult tissues under these conditions, we extended our observations by *in situ* hybridization on adult brain using an oligonucleotide specific for this cassette. Expression of the hip cassette was most prominent in the olfactory bulb and the hippocampus but at a lower level than the other receptor splice variants. This may indicate that the hip cassette is expressed at a low level in all cells expressing the PACAP receptor or is restricted to a subpopulation of neuronal or glial cells. Alternatively, it could be expressed in a time-restricted fashion during development.

Thus, we have isolated the PACAP-specific receptor using a novel expression cloning method for isolating cDNAs encoding proteins regulating the cAMP transduction pathway. Like other G-protein coupled receptors such as calcitonin, parathyroid hormone and thyrotropin receptors, the PACAP receptor is posi-

TABLE 1 EC₅₀ (nM) values for stimulation of intracellular cAMP and inositol phosphate production of all splice variants isolated

	cAMP		Inositol phosphates	
	PACAP-38	PACAP-27	PACAP-38	PACAP-27
PACAP-R	0.4	0.1	15	>1 μ M
PACAP-R-hip	6	1.7	NS	NS
PACAP-R-hop1	0.4	0.1	16	>1 μ M
PACAP-R-hop2	0.4	0.1	15	>1 μ M
PACAP-R-hip-hop1	2.5	0.7	50	>>1 μ M

PACAP-R-hop1 and -hop2 display similar results to those obtained with PACAP-R. The presence of the hip cassette in PACAP-R-hip or -hip-hop impairs both adenyl cyclase and phospholipase C stimulation. In the case of PACAP-R-hip no stimulation at all was observed for inositol phosphate production. Results give the mean of at least two experiments done in duplicate. NS, not stimulating at any dose tested. Methods were as described for Fig. 3a, b.

tively coupled to both adenylyl cyclase and phospholipase C. Experimental mutagenesis within the thyrotropin receptor revealed that the C-terminal part of the third intracellular loop is the key domain for coupling to phospholipase C via specific G protein(s)²⁶. Here we describe naturally expressed variants of this crucial domain allowing PACAP receptors to regulate cAMP or inositol phosphate levels differently. Furthermore,

we demonstrate that the same receptor can be differentially coupled to two transduction pathways through distinct natural ligands. This may indicate that coupling with a specific G protein can modify the pharmacology of the binding site or, more likely, that, depending on the molecular interactions within the binding site, preferential coupling with a specific G protein can occur. □

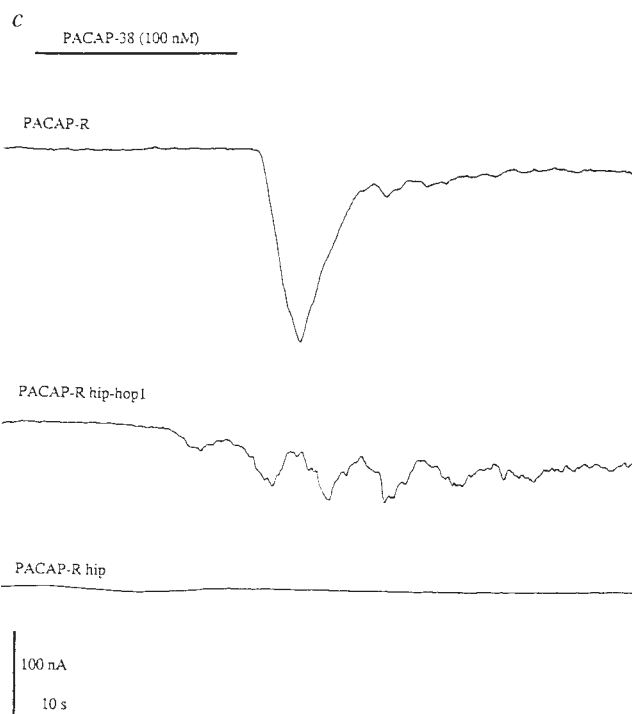
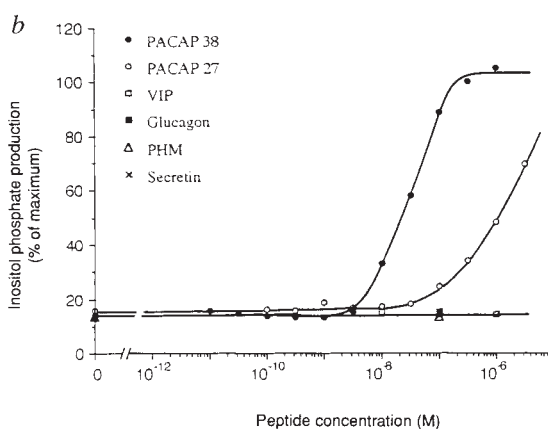
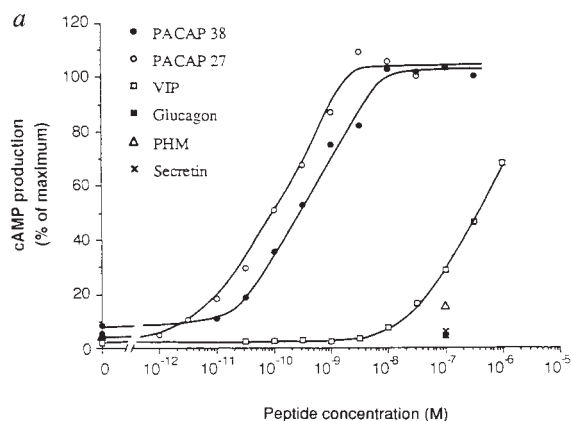
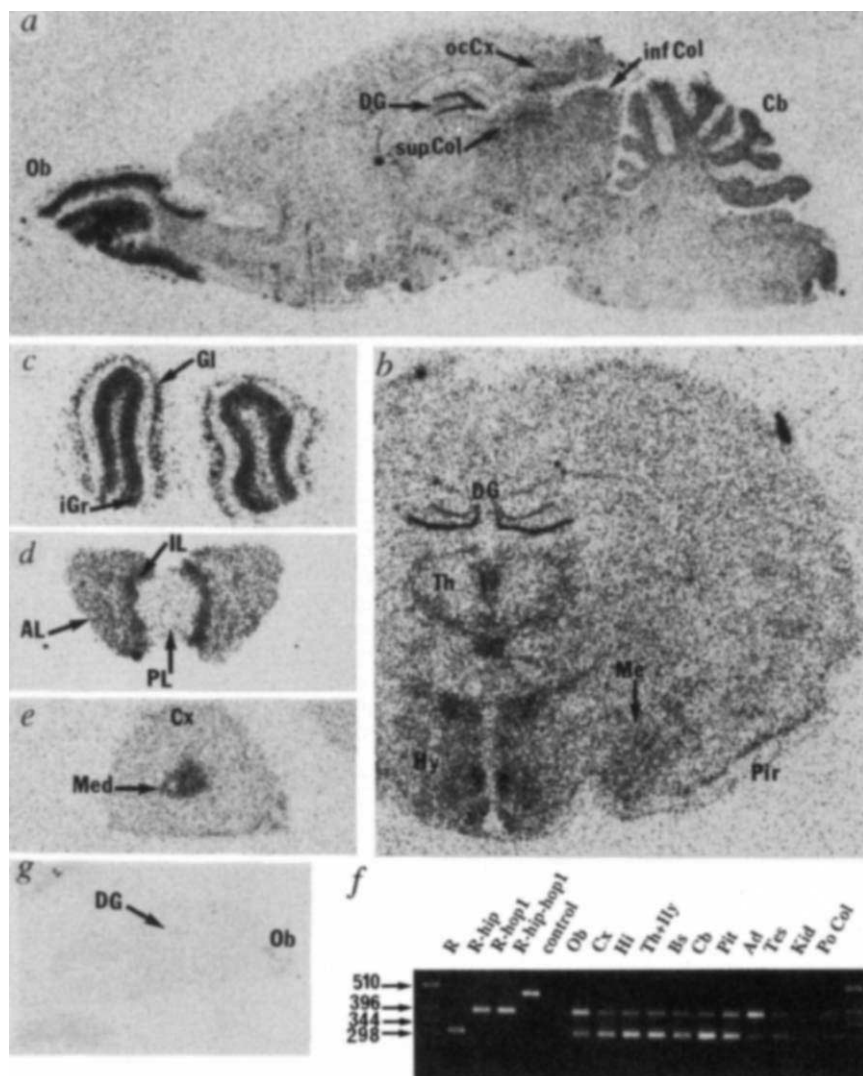


FIG. 3 Pharmacology of the splice variants of the PACAP receptor. *a*, Dose-response curves for stimulation of intracellular cAMP levels in LLC PK1 cells expressing the receptor PACAP-R. PACAP-38 and -27 have similar EC_{50} values (0.1–0.5 nM), whereas VIP is at least 1,000-fold less potent and related peptides of the same family are inactive at even the highest concentration tested (100 nM). *b*, Dose-response curves for inositol phosphate production on transfection of PACAP-R in LLC PK1 cells. PACAP-38 displays an EC_{50} of 15 nM whereas PACAP-27 is at least 100-fold less potent and related peptides are not active at all. *c*, PACAP-38 (100 nM, 25 s perfusion) induced membrane current recorded from voltage-clamped (–70 mV) *Xenopus* oocytes injected with *in vitro* transcribed RNA coding for PACAP-R (top trace), PACAP-R-hip-hop (middle trace), and PACAP-R-hip (bottom trace). At least eight recordings were obtained for each splice variant.

METHODS. *a*, *b*, LLC PK1 cells were electroporated with 0.1 μ g of receptor cDNA, aliquots were plated into 12-well clusters and [3 H]adenine or myo[3 H]inositol were added for 24 h. Incubations were done in duplicate for 10 min in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 100 μ M Ro20-1724 for stimulation of cAMP levels and for 20 min in PBS containing 10 mM LiCl for inositol phosphate production. Levels of cAMP and inositol phosphates were quantified as described previously^{31,32}. *c*, cDNAs were transcribed *in vitro* using SP6 polymerase and 10 ng RNA was injected into defolliculated oocytes. Recordings from oocytes were made in Barth's medium 3–4 days later using the two-electrode voltage-clamp technique (Axo-clamp-2A). Data were recorded on a PC computer using the PCLAMP software.

FIG. 4 Expression pattern of PACAP-receptor mRNA by *in situ* hybridization and RT-PCR. *In situ* experiments shown in a–e and g were performed with ^{33}P -labelled oligonucleotide probes PAC-IS1 (5'-CCGGTGCTTGAAGTCCATAGTGAAGTAACGGTTCACCTT-3'), a sequence present in all splice variants and PAC-IS2 (5'-GTCTGAGGGACACAGGCAGGGGGTCTCTCGGGTTTCTT-3') which is specific for the cassette hip. In control experiments, RNase pretreatment of sections abolishes the hybridization signal obtained with the probes. a, Parasagittal section of adult rat brain. Regions that express the PACAP receptor include olfactory bulb (Ob), dentate gyrus (DG), superior and inferior colliculi (supCol and infCol, respectively), occipital cortex (OcCx) and cerebellum (Cb). b, Coronal section of adult rat brain. Prominent expression is noted in dentate gyrus (DG), several nuclei of thalamus (paraventricular thalamus nucleus, lateral habenular nucleus, centrolateral thalamus nucleus, paracentral thalamus nucleus, central medial thalamus nucleus, rhomboid thalamus nucleus), several nuclei of hypothalamus (dorsal hypothalamus area, zona incerta, ventromedial hypothalamus nucleus, arcuate hypothalamus nucleus), medial amygdaloid nucleus (Me) and piriform cortex (Pir). c, Coronal section of the olfactory bulb. Expression is noted in the glomerular layer (Gl) and the intermediate granular layer (iGr). d, Section of adult pituitary. High levels of expression are seen in intermediate (IL) and anterior (AL) lobes, whereas much lower expression, if any, is noted in posterior lobe (PL). e, Section of the adrenal gland showing intense labelling of the adrenal medulla (Med) compared with the adrenal cortex (Cx). f, Molecular forms of PACAP receptor mRNA detected by RT-PCR of different tissues. A pair of oligonucleotides located 56 bp upstream and 247 bp downstream of the site of insertion of the cassettes was used for PCR on cDNA generated from total RNA of olfactory bulb (Ob), cerebral cortex (Cx), hippocampus (Hi), thalamus + hypothalamus (Th + Hy), brain stem (Bs), cerebellum (Cb), pituitary (Pit), adrenal gland (Ad), testes (Tes), kidney (Kid) and newborn rat colliculi (Po Col). Simultaneously, we did PCR on the cDNA clones encoding PACAP-R (R), PACAP-R hip (R-hip), PACAP-R hop1 (R-hop1), PACAP-R hip-hop1 (R-hip-hop1) and without matrix (control), which served as positive and negative controls, respectively. The PCR products of these reactions were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Arrows indicate the size of the marker fragments. The 303-bp PCR fragment corresponds to PACAP-R, whereas the 387-bp PCR fragment consists mainly of the hop isoform as verified by hybridization with specific oligo-



nucleotides (data not shown). g, *In situ* hybridization of parasagittal section of adult rat brain with the oligonucleotide PAC-IS2 specific for the hip cassette.

METHODS. *In situ* hybridization experiments were performed as previously described³³ except that [α - ^{33}P]dATP was used instead of [α - ^{32}P]dATP for labelling the oligonucleotide. The autoradiograms were exposed for 3–5 days. For RT-PCR experiments, single-stranded cDNAs were synthesized using H⁺MoMuLVRT (Gibco), primer NC3X and total RNA (1.0 μg). This template was used in amplification with primers 453-40P (5'-CTTGTACAGAAGCTGCAGTCCCCAGACATG3') and PAC-IS1 for 45 cycles (94 °C/30 s + 60 °C/30 s + 72 °C/45 s) or 60 cycles for testes. 10% of the PCR products were loaded on 1.5% agarose gel.

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A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum

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UBIQUITIN-conjugating enzymes function in selective proteolysis pathways and catalyse the covalent attachment of ubiquitin to proteolytic substrates¹⁻⁴. Here we report the identification of an integral membrane ubiquitin-conjugating enzyme. This enzyme, UBC6, localizes to the endoplasmic reticulum (ER), with the catalytic domain facing the cytosol. *ubc6* loss-of-function mutants suppress the protein translocation defect caused by a mutation in *SEC61*, which encodes a key component of a multisubunit protein translocation apparatus of the ER⁵⁻¹¹. The expression of the *sec61* mutant phenotype requires both the activity of UBC6 and its locali-

zation at the ER membrane. This suggests that UBC6 may mediate selective degradation of ER membrane proteins and that the protein translocation defect of *sec61* may be caused by proteolysis of components of a structurally distorted mutant translocation apparatus.

We have cloned from the yeast *Saccharomyces cerevisiae* a gene, designated *UBC6*, that encodes a novel ubiquitin-conjugating enzyme (*M_r* 28.4K; 250 amino acids; Fig. 1). The deduced amino-acid sequence of UBC6 predicts a carboxy-terminal membrane anchor¹² (Fig. 1a). As UBC6 co-purified with the microsomal fraction and was solubilized only by detergents but not by agents that strip peripheral proteins from membranes (Fig. 2a, b), we conclude that, unlike the previously identified ubiquitin-conjugating enzymes^{13, 18}, UBC6 is an integral membrane protein. Proteinase K sensitivity assays (Fig. 2c) further indicated that the enzymatic domain of the enzyme faces the cytosol. *In situ* immunofluorescence of cells with anti-UBC6 antibodies gave a (ring-shaped) perinuclear staining (Fig. 2d) which was indistinguishable from that of KAR2 (an ER protein), indicating that UBC6 localizes to the ER and possibly the nuclear envelope.

ubc6 null mutants (*ubc6Δ*) generated by gene disruption are viable and grow at wild-type rates. Because of the intracellular localization of the enzyme, we investigated whether *ubc6Δ* mutants may exhibit synthetic phenotypes in conjunction with mutants of the secretory pathway. In fact, we found that the *ubc6Δ* mutation suppressed the *sec61* mutant phenotype (Fig. 3a). *SEC61*, an essential gene, encodes a multispanning ER mem-

a

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-180 aaatttttggttatttgggttcagaaaaagccgaatcgattgcagagaacagaaggaac
-120 ttccgattatacaactttagagccgtgataaagaagactaccatcgcatatccagaaa
-60 agactttaaatattataactaaaaccgcatcgcaaatgcaacaagtacgtacaatagta

1 ATGGGTACAAAGCAGGCTCACAAGAGATTGACGAAGAGTACAAGTTGATGGTGAAAAC
MetAlaThrLysGlnAlaHisLysArgLysThrLysGluTyrLysLeuMetValGluAsn

61 CCTCCACCATATATTTCTTGTCTCCGCCCAACGAAGATAATATTTAGAGTGGCATATATT
ProProProTyrIleLeuAlaArgProAsnGluAspAsnIleLeuGluTrpHisTyrIle

121 ATCAGGAGCACTGCGGATACCTCTTACAAGGGCGGTCAATATCAGCGTACTTTAACTTTC
IleThrGlyProAlaAspThrProTyrLysGlyGlyGlnTyrHisGlyThrLeuThrPhe

181 CCGTCTGATTATCCATACAAACCACCGGCTATCAGAATGATAACACCGAATGGACGTTTC
ProSerAspTyrProTyrLysProProAlaIleArgMetIleThrProAsnGlyArgPhe

241 AAGCCCAACACAGCATTATGCTCTTCTATGAGTGATTACCCCTGATCTTGGAACTCCT
LysProAsnThrArgLeuCysLeuSerMetSerAspTyrHisProAspThrTrpAsnPro

301 GGCTGGTCTGTCTCAACCATTTTGAACGGGTTACTAAGTTTCATGACCACTGATGAAGCC
GlyTrpSerValSerThrIleLeuAsnGlyLeuLeuSerPheMetThrSerAspGluAla

361 ACGACAGGATCAATTACAACATCAGACCATCAGAAGAAGCATTAGCAAGAAATTCATA
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421 AGTTATAACACTTTTCAAAATGTTAGATTCAAATTTGATTTTCCGGAAGTTGTACAGGAA
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481 AATGTAGAGACATTAGAAAAGAGAAAATTTGGATGAGGGGATGCGGCAAAATACAGGTGAT
AsnValGluThrLeuGluLysArgLysLeuAspGluGlyAspAlaAlaAsnThrGlyAsp

541 GAAACAGAAGACCCCTTTTACAAGGCTCGCAAGGAAAAGTCATCTCGTTGGAGGAAAT
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601 CTAGACCCCTGAAGACAGAATACGGCTGAACAAGCGTTGACAAATCAGAAAATTAATTC
LeuAspProGluAspArgIleArgAlaGluGlnAlaLeuArgGlnSerGluAsnAsnSer

661 AAGAAAGATGGCAAGAACCTAATGATAGTTCTCTCAATGGTTTATATGGTATCGCTATT
LysLysAspGlyLysGluProAsnAspSerSerSerMetValTyrIleGlyIleAlaIle
(... AspSerSerGlySer*** ubc6Δ*)

721 TTTTGTGTTTGGTTGGCTTTTATGAAATGAAGccattaaatgaactaaacttagat
GluLeuValGlyLeuPheMetLys***

781 aaattttgacaacacagaataagaatcaatagattatataagacaaaacggatcttttta
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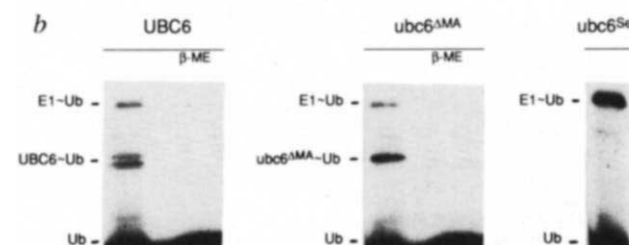


FIG. 1 UBC6 encodes a ubiquitin-conjugating enzyme. a, Nucleotide sequence of the *UBC6* gene and predicted amino-acid sequence of the encoded protein (EMBL accession no. X73284). The active-site cysteine residue of UBC6 required for thioester formation with ubiquitin is shown in bold. The presumed signal anchor sequence of UBC6 is underlined. The triangle denotes the position of the inserted HA1 epitope. The amino-acid sequence at the C terminus of the truncated protein *Ubc6^{ΔMA}* is shown in brackets. Nucleotide numbers starting at the first nucleotide of the coding region are given on the left. The asterisks represent stop codons. b, The UBC6 protein is an active ubiquitin-conjugating enzyme. A characteristic feature of ubiquitin-conjugating enzymes is their capacity to form a ubiquitin-enzyme thioester intermediate in the presence of ubiquitin-activating (E1) enzyme and ATP (refs 1-4). Extracts of *Escherichia coli* cells expressing (from left to right) UBC6, *ubc6^{ΔMA}* and *ubc6^{Ser87}* were incubated with yeast E1 enzyme, [¹²⁵I]-labelled ubiquitin and ATP. Gel electrophoresis followed by autoradiography detects thioester-linked complexes of labelled ubiquitin (Ub) with E1 (E1~Ub), UBC6 (UBC6~Ub) and truncated *ubc6^{ΔMA}*, (*ubc6^{ΔMA}*~Ub). Under reducing conditions (β-mercaptoethanol; β-ME) these complexes disappear, confirming the thioester linkage. Because of an unusual migration behaviour of UBC6 in SDS gels (similar to other ubiquitin-conjugating enzymes¹⁶; S. J., unpublished results), the UBC6-ubiquitin thioester runs as a doublet and the truncated *Ubc6^{ΔMA}* exhibits no increased mobility. The active-site mutant protein, *Ubc6^{Ser87}*, is unable to form a thioester with ubiquitin, confirming that it is an inactive protein.

METHODS. UBC6 was identified in a λEMBL3a yeast genomic DNA library with a 24-mer oligonucleotide (5'-GATATCCA/CTT/AC/TAAIC-CACCA/GA/CAG, where I represents inosine) corresponding to a conserved domain of ubiquitin-conjugating enzymes (roughly DYPFKPPK; single-letter code). For the construction of the mutant *ubc6^{Ser87}*, the cysteine codon (TGC) was changed into a serine codon (AGC). Mutant *ubc6^{ΔMA}* was constructed by a PCR-mediated fusion of UBC6 at codon 231 with the 3'-untranslated region of the UBC6 gene that followed the authentic stop codon of UBC6. The DNA sequence at the new 3'-end of the truncated gene (*ubc6^{ΔMA}*) is 5'-GAT-AGTTCTGGATCCTGAAGCCATTA (a). Expression of wild-type and mutant UBC6 proteins in *E. coli*, preparation of proteins, gel electrophoresis (18% polyacrylamide-SDS gel), enzyme assays and DNA techniques were all carried out as described^{13,16,28}.

brane protein required for the translocation of proteins across the ER membrane^{5,7}. The SEC61 protein is part of a multisubunit protein complex (presumed translocation channel) that also includes SEC62, SEC63 and additional proteins^{6,11}. At the restrictive temperature of the conditional lethal *sec61* mutant, translocation of proteins across the ER is strongly reduced and precursor proteins accumulate^{5,11,19}. Figure 3a shows that viability of *sec61* cultures at the restrictive temperature was restored in a *ubc6Δ* background (about 180 min generation time compared to 120 min of wild-type at 37.5 °C in SD media). But, neither the lethality of the *sec61* deletion mutant⁷ nor the phenotypes of *sec62* and *sec63* mutants¹¹ were suppressed by the *ubc6Δ* mutation, indicating that the suppression is specific for defects caused by mutant sec61 proteins.

To test whether protein translocation is also restored by *ubc6* loss-of-function mutants, cells were labelled with ³⁵S-methionine and proteins were immunoprecipitated with antibodies specific for KAR2 (an ER luminal protein¹⁹), carboxypeptidase Y (CPY, a protein of the vacuole) and α -factor (a secreted protein). In contrast to *sec61* cells, no precursor forms of KAR2 accumulated in *sec61 ubc6Δ* double mutants and the levels of the precursors of CPY and α -factor were significantly reduced (Fig. 3b). Moreover, we observed a significant increase of the glycosylated forms of CPY and α -factor in *sec61 ubc6Δ* cells (Fig. 3b), indicating that the translocation of both test proteins was restored to near wild-type levels.

We used two other *ubc6* mutant alleles in similar assays. One

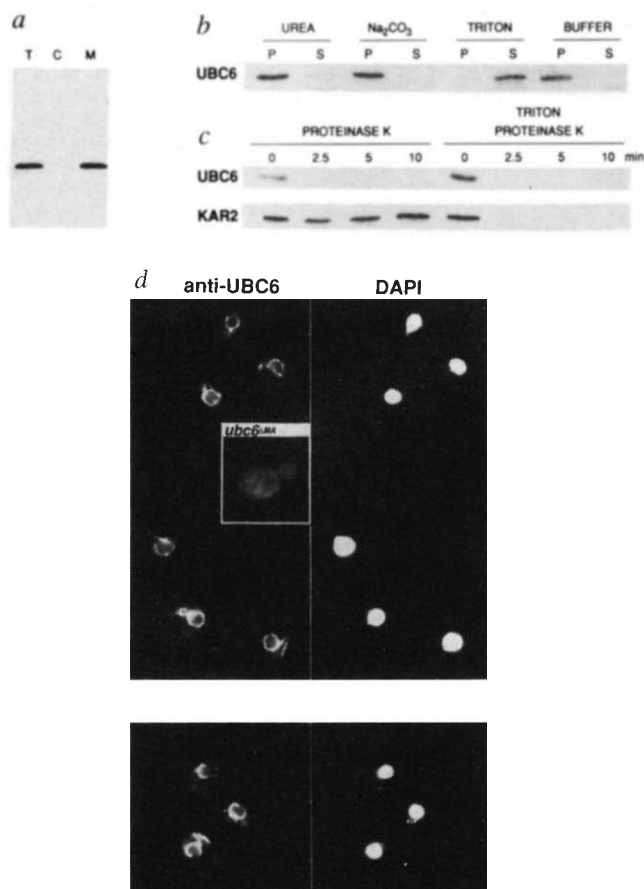
mutant, *ubc6^{Ser87}*, expresses an inactive UBC6 protein (Fig. 1b) in which the active site cysteine is replaced by serine. The other, *ubc6^{ΔMA}*, expresses an enzymatically active, but soluble enzyme (Fig. 1 and Fig. 2d inset; protein level similar to wild-type UBC6) that lacks the C-terminal membrane anchor (MA). As both mutant alleles were found to suppress *sec61* phenotypes equally well as the *ubc6* deletion mutant (Fig. 3a), we conclude that the expression of the *sec61* mutant phenotype requires an enzymatically active and membrane-bound UBC6 enzyme, and that the crucial substrates of UBC6 are most probably ER-membrane proteins.

Strong overproduction of UBC6 (about 20-fold) led to a weak suppression of the *sec61* phenotype (generation time of 300 min versus 180 min of *sec61 ubc6Δ*; see above). Yet UBC6 overexpression only partially suppressed the translocation defect of KAR2 and it had no effect on the translocation of CPY or α -factor (not shown). Importantly, suppression of *sec61* by UBC6 overexpression also occurred with an enzymatically inactive UBC6 protein (Ubc6^{Ser87}; data not shown), suggesting that this suppression is mediated by a titration effect. As some ubiquitin-conjugating enzymes must form tight complexes with specific substrate recognition (E3) proteins of the ubiquitin system in order to be active on certain substrates^{2,20}, overproduced membrane-bound UBC6 may sequester a limiting E3 (away from the substrate), leading to a reduced capacity to ubiquitinate relevant substrates.

Recent evidence indicates that, like other ubiquitin-conjugat-

FIG. 2 UBC6 is an integral membrane protein of the ER. a, UBC6 fractionates with yeast microsomes. Immunoblot showing that monoclonal HA1-epitope antibodies (12CA5; ref. 29) react in a yeast whole-cell extract (T, total) with HA1-tagged UBC6 (lane 1). Cell fractionation shows that this protein is present in the microsomal fraction (M) but not in the supernatant containing cytosol (C). b, UBC6 is an integral membrane protein. Microsomes were treated with 2.5 M urea, 0.1 M Na₂CO₃, 0.5 M NaCl (not shown), 1% Triton X-100 or buffer, and soluble (S) and pellet (P) fractions (containing microsomes) were separated by centrifugation and probed in a western blot with anti-UBC6 antibodies. UBC6 can only be solubilized by Triton but not by agents that strip peripheral proteins from membranes. c, The catalytic domain of UBC6 faces the cytosol. Intact yeast microsomes were treated with proteinase K in the absence (left) or presence (right) of 0.4% Triton X-100 (microsome disruption) for the times indicated and samples were probed as above. UBC6 crossreactivity disappears rapidly in the absence or presence of Triton. In contrast, the ER-luminal protein KAR2, is only sensitive to proteinase K after treatment with Triton, confirming that the vesicles are inside-in. d, Intracellular localization of UBC6. Indirect immunofluorescence with anti-UBC6 antibodies shows a perinuclear staining (ring-shaped image) with occasional thin filaments extending into the cytosol and to the plasma membrane. Staining with DAPI shows the position of the nucleus. The image obtained with anti-UBC6 antibodies is indistinguishable from that with anti-KAR2 antibodies, suggesting that UBC6 localizes to the ER (and possibly the nuclear envelope). The inset shows that yeast cells expressing the truncated *ubc6^{ΔMA}* protein that lacks the C-terminal membrane anchor give a cytosolic staining with anti-UBC6 antibodies.

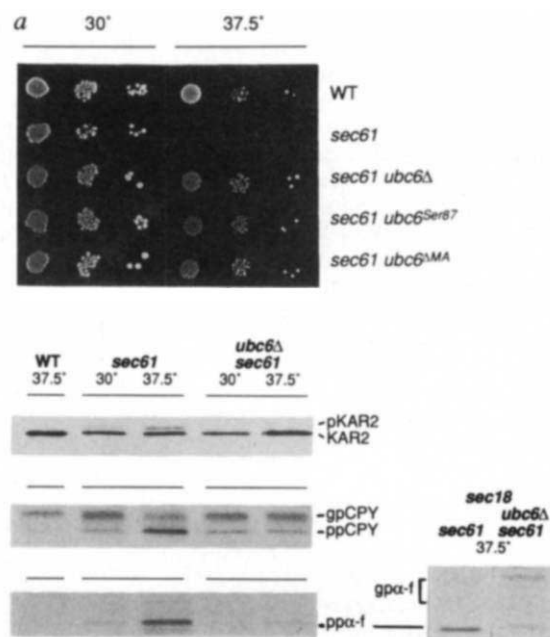
METHODS. UBC6 was tagged with the HA1-epitope of haemagglutinin²⁹ by insertion of a HA1-encoding oligonucleotide at the XbaI site at codon 202 (Fig. 1a, triangle). The resulting amino-acid sequence is LVYP-YDVPDYASLD (HA1 epitope in italics). Yeast cells expressing the tagged UBC6 protein (pSEY8 plasmid²⁸) were used for the preparation of yeast microsomes as described³⁰. The tagged protein is a functional UBC6 as indicated by its capacity to mediate the *sec61* phenotype (not shown). Fractions containing intact microsomal vesicles were prepared³⁰ (strain YW01 (ref. 16) expressing UBC6 from a pSEY8-based plasmid) and incubated for 30 min at 0 °C with the agents described in Fig. 2b. Vesicle-containing fractions and soluble fractions were separated as described¹⁹ and UBC6 was detected in western blots using anti-UBC6 antibodies (unpublished results). Similar microsomal crude fractions were treated with proteinase K (0.3 mg ml⁻¹) for the times indicated (c) with or without 0.4% Triton X-100. Aliquots were precipitated with 20% trichloroacetic acid and probed with anti-UBC6 and anti-KAR2 (obtained from M. Rose) antibodies in western blots. Indirect immunofluorescence



on permeabilized diploid yeast cells using anti-UBC6 and anti-KAR2 antibodies (secondary FITC-conjugated antibodies, Dianova) and DAPI (Sigma) staining was done as described³⁰. Strain YTX16 (diploid DF5 (ref. 16) bearing a homozygous *ubc6::HIS3* knock out; see below) transformed with a UBC6-expressing pSEY8 plasmid was used for UBC6 and KAR2 detection. For *ubc6^{ΔMA}* detection we used YTX16 transformed with a *ubc6^{ΔMA}*-expressing pSEY8 plasmid.

FIG. 3 *ubc6* loss-of-function mutants are suppressors of the yeast secretion mutant *sec61*. **a**, Suppression of *sec61* growth defects. Wild-type (WT), *sec61* mutant (*sec61-2*), and *sec61* mutants expressing either no UBC6 (*ubc6Δ*) or an inactive UBC6 protein (*ubc6^{Ser87}*) or a soluble UBC6 enzyme lacking the C-terminal membrane anchor (*ubc6^{ΔMA}*) were plated in three 10-fold dilutions on SD plates and incubated for three days at either 30 °C or 37.5 °C (restrictive temperature of *sec61-2*). All three *ubc6* mutants suppress the *sec61* growth defect equally well, indicating that UBC6 must be active and ER-membrane-bound in order to mediate the *sec61* mutant phenotype (both *sec61-2* and *sec61-3* alleles are suppressed, not shown). **b**, Suppression of *sec61* protein translocation defect. Wild-type (WT), *sec61* mutant (*sec61-2* allele) and *sec61 ubc6Δ* double mutant cultures were labelled with ³⁵S-methionine either at 30 °C or 37.5 °C (15 min) and proteins were immunoprecipitated with antibodies specific for KAR2, CPY and α -factor (α -F). In *sec61* cells at 37 °C (also slightly at 30 °C) translocation of these proteins is affected and unprocessed precursors of KAR2 (pKAR2), CPY (ppCPY) and α -factor (pp α -F) accumulate. Precursor accumulation is absent (KAR2) or reduced (CPY, α -F) in the absence of UBC6 (*ubc6Δ*). Translocation of CPY in the *sec61 ubc6Δ* strain (we used *pep4* mutant strains to prevent the formation of mature CPY that migrates close to preproCPY) is indicated by the increase of the fully glycosylated precursor of CPY (gpCPY). Likewise, translocation of α -factor (bottom right) across the ER membrane occurs in the *sec61 ubc6Δ* double mutants (demonstrated in strains with additional *sec18* mutations to visualize the ER-form of secretory proteins), as shown by the increase of core glycosylated α -factor precursor (gp α -F) in the absence of UBC6.

METHODS. *UBC6* gene disruption was done by deleting the coding region between nucleotide -11 and 604 (see Fig. 1 for numbering) followed by replacement with either a 2.1 kb *LEU2* fragment²⁸ (*ubc6::LEU2*) or a 1.8 kb *HIS3* fragment²⁸ (*ubc6::HIS3*). Mutants obtained with both types of constructs are phenotypically indistinguishable. For the construction of *ubc6* mutants linear DNA of the *ubc6::LEU2* plasmid was used for transformation of wild-type strain DF5 (ref. 16), *sec61* strains (*sec61-2* allele YFP338; ref. 19; *sec61-3* allele RSY455, provided by R. Schekman), *sec62* and *sec63-1* strains (RSY529 and RSY151; R. Schekman), diploid heterozygote *sec61* knock out strain (*SEC61/sec61::HIS3*; strain RSY456; R. Schekman), and a *sec61 sec18-1* double mutant strain (obtained from a cross of YFP338



with HMSF176; Yeast Genetic Stock Center, Berkeley). Strain YFP338 is mutant for *pep4*, allowing the detection of ppCPY. A homozygous *ubc6::HIS3* knock-out in diploid DF5 (strain YTX16) was used for the experiment shown in Fig. 2d. Homologous recombination at *UBC6* was verified by DNA hybridization. For the expression of mutant Ubc6 proteins (Fig. 3a) we used *sec61 ubc6Δ* double mutants (YFP338 with *ubc6::LEU2*) transformed with low copy number (*ARS1 CEN*) plasmids (pDP83; ref. 28) expressing either *ubc6^{Ser87}* or *ubc6^{ΔMA}* (UBC6 expressed from pDP83 does not suppress *sec61*). Standard protocols were used for preparation of media (YPD and SD), cloning and yeast techniques^{28,30}. Protein translocation assays were done essentially as described¹¹.

ing enzymes, UBC6 functions in proteolysis pathways²¹. We assume that substrates of UBC6 include membrane proteins and that the protein translocation defect of *sec61* is mediated by proteolysis of subunits of the mutant translocation apparatus (Fig. 4). As the mutant Sec61 protein appears to be a stable protein in *sec61* cells (not shown), candidate substrates for UBC6-mediated degradation are proteins which directly associate with Sec61 in a structurally distorted (misassembled) translocation apparatus. In the absence of UBC6, these subunits are not degraded and proteins can be translocated across the ER membrane (though at a reduced rate) using the mutant translocation apparatus. We propose that degradation of ER membrane proteins is a general function of the ubiquitin system and that this pathway may be related to the proteolysis system of the mammalian ER ('ER degradation')^{22,23}. Targets of ER degradation include specific subunits of aberrantly assembled T-cell receptors and asialoglycoprotein receptors^{22,23} and a mutant

temperature-sensitive form of the cystic fibrosis transmembrane conductance regulator (CFTR)²⁴. The regulated degradation of hydroxymethylglutaryl-CoA (HMG-CoA) reductase and of the membrane-bound form of apolipoprotein B is also thought to be mediated by a similar system^{22,23}. Further support for a role of the ubiquitin system in ER degradation (for example for membrane proteins with cytosolic domains) comes from findings that the proteasome that degrades ubiquitin-protein conjugates is associated with the ER membrane²⁵, and that the short-lived form of cytochrome P-450 (an ER membrane protein) appears to be ubiquitinated²⁶. The lack of deleterious *ubc6* mutant phenotypes suggests that for the degradation of the majority of ER membrane proteins, functionally overlapping pathways may exist. Perhaps one alternative path is lysosomal (vacuolar) degradation, because the default compartment for membrane proteins in yeast appears to be the vacuole²⁷. It will be important to identify the substrates of the UBC6 enzyme that will allow us

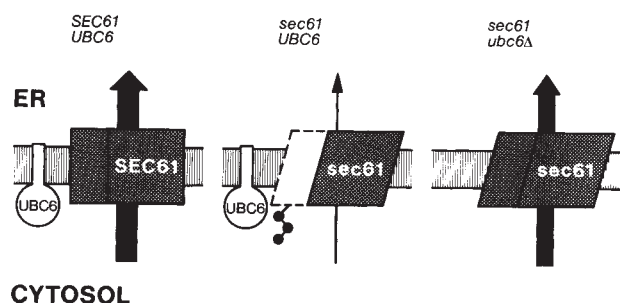


FIG. 4 Model for UBC6 function in *sec61* cells (from left to right). Protein translocation (thick arrow) across the ER membrane (striped horizontal bar) in wild-type cells (left) requires a multisubunit translocation apparatus (large shaded box) consisting of SEC61, SEC62, SEC63 and additional subunits. Only SEC61 and a putative UBC6 substrate (left segment of the box) are shown. In *sec61* cells at the non-permissive temperature (centre) the translocation apparatus is structurally distorted (represented by the tilted conformation), leading to a UBC6-catalysed ubiquitination (chain of filled circles) of one of its subunits and its subsequent degradation (dashed white segment). Partial proteolytic elimination of a subunit of the translocation apparatus results in the *sec61* translocation defect (strongly reduced protein translocation; thin arrow). In the absence of UBC6-mediated degradation (*sec61 ubc6Δ* double mutant; right) protein translocation is restored to close to wild-type levels.

to address the mechanisms of substrate selectivity of this system. We have recently cloned several high-copy-number suppressors of *sec61* which may encode those substrates. □

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c-Jun is essential for normal mouse development and hepatogenesis

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THE proto-oncogene *c-jun* is the cellular homologue of *v-jun*, the transforming oncogene of the avian sarcoma virus 17 (ref. 1). *c-jun* encodes one major component of the AP-1 transcription factor complex and is expressed in many organs during mouse development and in the adult^{2–4}. Because of its rapid induction in cells following growth stimulation and the presence of AP-1 binding sites in the promoter regions of many genes, the c-Jun protein is thought to have important functions in cell proliferation and differentiation^{2,5–10}. But embryonic stem (ES) cells lacking c-Jun are viable and have a normal *in vitro* differentiation capacity, although c-Jun appears to be important for growth of teratocarcinomas *in vivo*¹¹. To define the function of *c-jun* better, targeted ES cells were used to generate mice lacking c-Jun. Here we report that heterozygous mutant mice appear normal, but embryos lacking c-Jun die at mid- to late-gestation and exhibit impaired hepatogenesis, altered fetal liver erythropoiesis and generalized oedema. Interestingly, *c-jun*^{−/−} ES cells can participate efficiently in the development of all somatic cells in chimaeric mice except liver cells, further suggesting an essential function of c-Jun in hepatogenesis.

We previously generated D3 ES cell clones containing one disrupted *c-jun* allele¹¹. Using the D3-2 ES cells we have now generated mice heterozygous for the mutated *c-jun* allele which transmitted the disrupted allele to their offspring (Fig. 1a). These mice appeared normal and were then intercrossed. Out of 63 pups, no homozygous *c-jun*^{−/−} mice were born, implying that *c-jun*^{−/−} embryos die *in utero* (Table 1).

To investigate the timing of this embryonic lethality, 231 embryos from day 8.5 to 16.5 post coitum were collected and genotyped (Table 1). No statistically significant differences were observed in the number of *c-jun*^{−/−} embryos obtained before day 10.5, when compared with the expected mendelian ratio ($\chi^2 = 2.09$, $P > 0.05$), although we cannot exclude the possibility that

TABLE 1 Viability of *c-jun*^{−/−} embryos

Age	Number of litters	Number of pups	Genotype of live embryos*		
			+/+	+/-	-/-
E8.5–10.5	8	64	18	35	11
E11.5–12.5	11	85	20	42	8
E13.5–15.5	9	61	11	36	5
E16.5	3	21	6	14	0
W1	12	63	21	35	0

Analysis of *c-jun* genotypes in offspring generated from heterozygote intercrosses. DNA was isolated from embryos between days 8.5 and 16.5 of gestation (E8.5–E16.5) and from 1-week-old (W1) mice and analysed by Southern blots. Note the decrease in the proportion of live *c-jun*^{−/−} embryos after E10.5. +/+, Wild-type; +/-, heterozygotes; -/-, homozygotes.

* In addition to the number of live embryos, numerous resorptions were also observed between days E11.5 and E16.5, some of which were homozygous mutants.

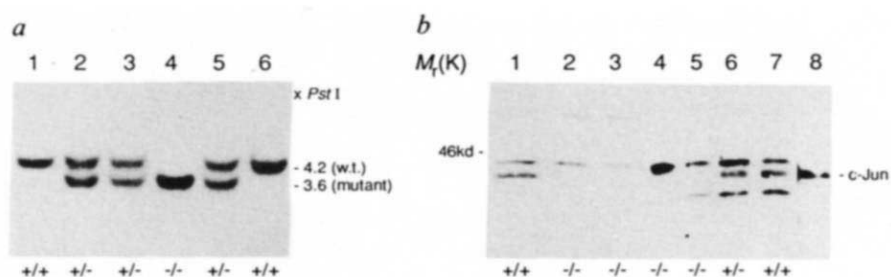
some may die earlier. The proportion of live *c-jun*^{−/−} embryos decreased, with the time of death varying between days 11.5 and 15.5 (Table 1). The absence of c-Jun protein was confirmed in day 9.5 *c-jun*^{−/−} embryos by western blot analysis using a c-Jun-specific antibody (Fig. 1b).

Histological analysis of homozygous mutant embryos at different stages of development revealed that 5 of 8 embryos at day 12.5 and 2 out of 2 embryos at days 13.5–14.5 had morphological anomalies (Fig. 2a). Tissues were oedematous, most prominently in the brain, as seen by the large intercellular spaces (Fig. 2c). In the liver, the epithelial compartment was hypoplastic and the residual hepatocytes visualized by keratin immunostaining were rounded and detached from each other (Fig. 2e, g). Scattered cells often displayed apoptosis or necrosis and no developing ducts were observed. In control littermates arrays of coherent polygonal cells along the sinusoidal linings could be discerned (Fig. 2d, f). Extensive hepatic erythropoiesis was prominent in *c-jun*^{−/−} embryos with large clusters of benzidine-positive nucleated erythroid cells (Fig. 2i). Nuclear accumulation of proliferating cell nuclear antigen immunoreactivity was found in several cells, suggesting that they were proliferating (Fig. 2j, k). One embryo of this group at day 12.5 had grossly congested vessels and signs of early liver damage. A large number of cells had a small hyperchromatic nucleus with fragmented chromatin, suggesting apoptosis. In another case, small nests of necrotic cells were randomly distributed throughout the body, indicating possible metabolic damage and imminent death of the embryo. *c-jun*^{−/−} embryos aged 13.5 days and older were frequently found dead *in utero*. Although oedema often deformed these embryos,

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FIG. 1 Genotype identification and c-Jun protein expression in embryos derived from heterozygous intercrosses. **a**, Southern blot analysis of *Pst*I-digested DNA isolated from 6 littermates at 10.5 days of gestation. The 4.2-kilobase (kb) fragment represents the endogenous wild-type *c-jun* allele (w.t.) and the 3.6-kb band the mutant *c-jun* allele (see also ref. 11). The genotype at the *c-jun* locus is indicated below each lane. **b**, Western blot analysis of c-Jun protein expression in E9.5 embryos and in doubly targeted *c-jun*^{-/-} ES cells. Protein lysates (50 µg per lane) from wild-type (lane 1) and 2 independent *c-jun*^{-/-} ES cell clones (lanes 2 and 3), 2 homozygous (lanes 4 and 5), 1 heterozygous (lane 6), 1 wild-type embryo (lane 7) and 10 ng bacterially expressed recombinant c-Jun protein (lane 8) were separated on an SDS-PAGE gel, blotted onto nitrocellulose and analysed with an anti-c-Jun rabbit polyclonal antibody. The arrow indicates the p39 c-Jun protein; the 2 additional bands reflect nonspecific background.

METHODS. Chimaeric mice were generated by microinjection of D3-derived D3-2 and D5-1 ES cells (*c-jun*^{-/-}; 11) into C57BL/6 blastocysts. Several chimaeric males generated with D3-2 cells transmitted the targeted *c-jun* allele to their offspring. Mice heterozygous for the *c-jun* mutation were intercrossed to generate homozygous mice at the mutated locus. Some of these mice result from matings of chimaeras to 129/Sv females and therefore had a homogeneous genetic background, whereas others were generated from matings to C57BL/6 females resulting in a heterogeneous genetic background. For genotyp-



ing, DNA was prepared from embryos and analysed by Southern blotting as previously described¹¹. For c-Jun protein analysis, mouse embryos were removed and frozen. The frozen tissue was homogenized and lysed in ECB buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40 containing 50 µg ml⁻¹ leupeptin and 100 µg ml⁻¹ phenylmethylsulphonyl fluoride). ES cells were treated for 1 h with TPA (100 ng ml⁻¹), washed in PBS and lysed in ECB buffer on ice. Protein concentration in the lysates was quantified by the Bradford method²⁰. The recombinant c-Jun protein was provided by M. Karin (UCSD). Equivalent amounts of protein (50 µg) were separated on an SDS-PAGE gel and blotted onto an Immobilon membrane (Schleicher & Schüll); the membrane was blocked with 10% milk in PBS and incubated with a polyclonal anti-c-Jun antibody¹¹. After washing the membrane four times with 5% milk and 0.05% Tween-20 in PBS the antibody was visualized using the ECL detection system (Amersham).

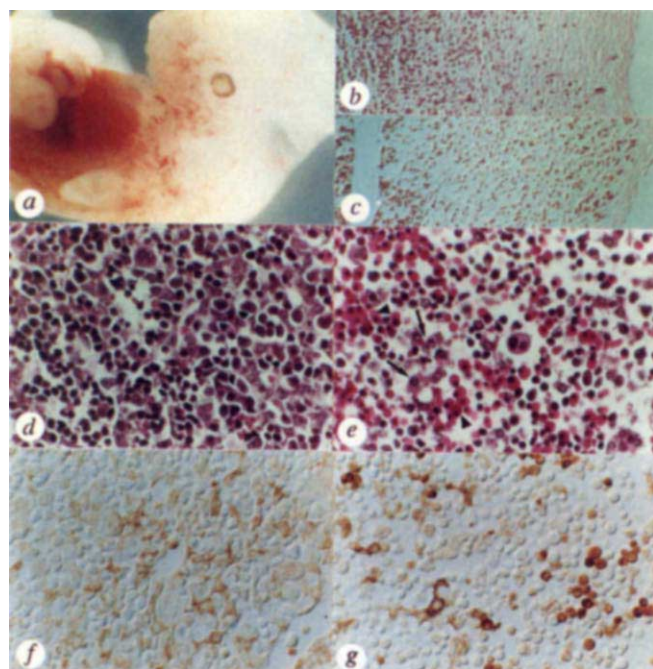
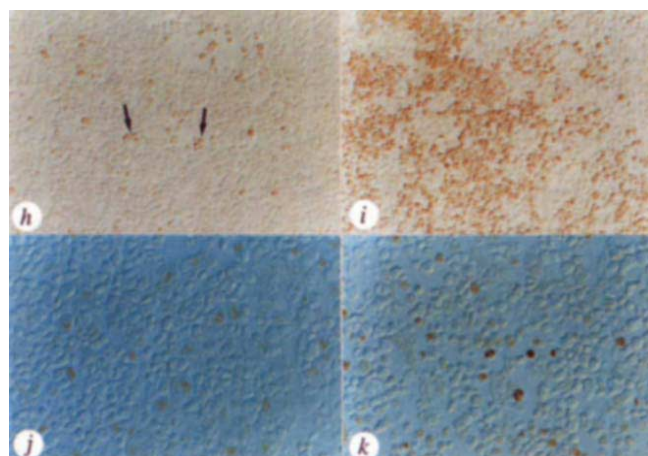


FIG. 2 Morphological appearance of *c-jun*^{-/-} and *c-jun*^{+/-} embryos at 12.5 days of gestation. **a**, *c-jun*^{-/-} embryo showing excessive quantity of blood in tissues. **b**, Forebrain of a *c-jun*^{+/-} embryo showing normal development. **c**, Forebrain of a *c-jun*^{-/-} embryo suffering from severe oedema as shown by the large intercellular spaces. Despite the obvious morphological changes, the number of proliferating cell nuclear antigen-labelled cells is comparable in *c-jun*^{+/-} and *c-jun*^{-/-} embryos, suggesting that these changes preceded proliferative impairment (data not shown). **d**, A liver of a *c-jun*^{+/-} embryo showing balanced relation of hepatocytic and haematopoietic differentiation (×164). **e**, A liver of a *c-jun*^{-/-} littermate showing dissociated hepatocytes (arrows) and enhanced erythropoiesis with increased numbers of nucleated erythrocytes (arrowheads) (×164). **f**, Keratin immunostaining of a *c-jun*^{+/-} liver showing moderate cytoplasmic immunoreactivity of hepatocytes with



intact cell connections (×164). **g**, Keratin immunostaining of a *c-jun*^{-/-} liver. A positive signal is seen in dissociated, mostly rounded cells (×164). Almost no epithelial linings are observed. **h**, Benzidine histochemistry of a *c-jun*^{+/-} liver showing occasional erythroid cells (arrow, ×82). **i**, Benzidine histochemistry of a corresponding *c-jun*^{-/-} liver showing greatly enhanced erythroid differentiation (×82). **j** and **k**, PCNA immunostaining of embryonic *c-jun*^{+/-} and *c-jun*^{-/-} livers, respectively. Although both hepatocytes and haematopoietic cells are stained in the *c-jun*^{+/-} liver, nuclear labelling is present in rounded cells in the *c-jun*^{-/-} liver.

METHODS. Seventeen embryos at 11.5–15.5 days of gestation were embedded in paraffin, cut in sagittal serial sections of 4 µm and stained with haematoxylin/eosin and with benzidine. Immunostains included proliferating cell nuclear antigen (Dianova, Hamburg) and keratins²¹.

no malformations could be identified histologically in addition to those described above. In particular, formation of cartilage did not appear to be affected, which is surprising because AP-1 is thought to have an important role in cartilage differentiation^{3,12}.

To investigate a possible cell-autonomous effect of *c-Jun* on organogenesis, two independent ES cell clones lacking *c-Jun* (D3-2/2-3 and D3-2/4-1, both obtained by selection of clone D3-2 in high concentrations of G418) were used to generate chimaeras. The absence of functional *c-Jun* in these clones was verified by western blot analysis (Fig. 1b). Most chimaeras showed high ES cell contribution (as judged by the agouti coat colour) and both clones contributed to gametogenesis as indicated by germ-line transmission. The degree of ES cell contribution to various organs of four chimaeric mice was analysed by glucose phosphate isomerase isozyme analysis. Figure 3 shows efficient ES cell contribution to all organs with the exception of liver, although other endoderm-derived tissues like lung and intestine contained up to 50% ES cell derivatives. The parental *c-jun*^{+/−} clone (D3-2) showed ES cell contribution to all organs including the liver in two chimaeric males (data not shown). These results demonstrate that whereas *c-Jun* is not required for the differentiation of most cell types including sperm, it is apparently essential for normal hepatogenesis.

To confirm that *c-jun* is solely responsible for the observed embryonic lethality we attempted to rescue the genetic defect with a *c-jun* transgene. Transgenic mice expressing *c-jun* efficiently from an H2 promoter exhibit no apparent phenotype¹³. These were crossed to *c-jun*^{+/−} mice and offspring were subsequently intercrossed to yield mice homozygous for the inactivated *c-jun* allele, but containing several copies of the transgene (J. Liang, unpublished results). The mice are viable and some have reached 3.5 months with no obvious abnormalities (data not shown), demonstrating that the embryonic lethality is indeed caused by the lack of functional *c-Jun*. A similar rescue was reported for mice lacking the retinoblastoma (*Rb-1*)

gene¹⁴. *Rb-1* mutant embryos die at a similar stage and show defects in neurogenesis and haematopoiesis^{14,16}; however, in contrast to *c-jun*, no cell-autonomous deficiency of *Rb-1*^{−/−} cells could be described (A. Berns, personal communication).

This work demonstrates that although inactivation of one *c-jun* allele in mice has no effect on embryogenesis, homozygous mice fail to develop to term, indicating an essential function for *c-Jun* in fetal development. This differs from the reported data for *c-fos*, the other major component of the AP-1 transcription factor complex^{17,18}. In contrast to ES cells lacking *c-Jun*, in which other members of the *c-jun* family such as *junB* may substitute some *c-jun* functions¹¹, no apparent compensation occurs in *c-jun*^{−/−} embryos. The broad expression of *c-jun* during organogenesis, in particular in developing cartilage, gut and the central nervous system³, does not explain the observed tissue specificity described here, although the expression pattern of *c-jun* embryos before day 14.5 was not analysed³. The death range in the embryos may be attributed to a variable expressivity of *c-Jun*, as has been suggested for other developmental control genes¹⁹. It is also possible that metabolic liver failure occurs in *c-jun*^{−/−} embryos and that death may ensue at different points of gestation in the absence of prominent malformations. In addition, the accumulation of toxic metabolites may be responsible for the observed generalized oedema and the areas of necrosis which are often seen in the late phases of the phenotype. *c-jun*^{−/−} ES cells can differentiate efficiently into functional germ cells as well as into all somatic cells, with the exception of hepatocytes, yet fetal liver cells are formed transiently in homozygous mutant embryos derived from intercrosses. In chimaeras, *c-jun*^{−/−} ES derivatives may initially colonize the fetal liver, but encounter a selective disadvantage in comparison to host cells at later stages, whereas in homozygous mutant embryos this competition does not occur and the observed defective fetal hepatogenesis and the altered erythropoiesis could be explained by deregulated expression of a specific hepatocyte growth factor. The absence of haematopoietic pathologies in the chimaeras (despite substantial contribution of *c-jun*^{−/−} ES derivative to blood and bone marrow) suggests that the erythroblastosis-like phenomena detected in homozygous mutant embryos may represent a consequence of a preceeding pathology rather than a direct effect of the absence of *c-Jun*. Nevertheless, the apparent cell-autonomous defect of *c-jun*^{−/−} cells to form hepatocytes can be used to study the role of *c-Jun* and AP-1 in this differentiation pathway. □

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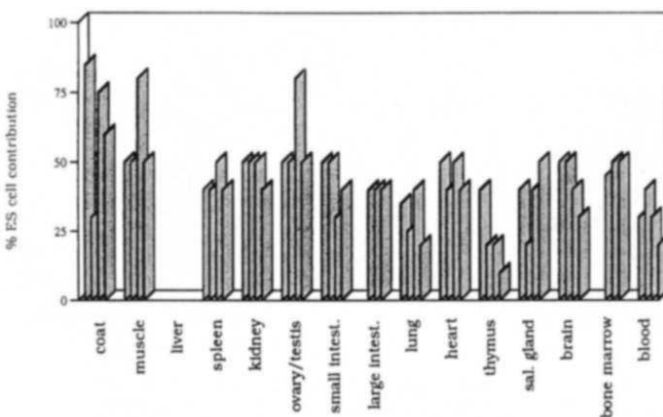


FIG. 3 Contribution of *c-jun*^{−/−} ES cell derivatives to different tissues of chimaeric mice. Four different chimaeras were analysed by glucose phosphate isomerase (GPI) isozyme assay to determine the ES cell contribution in various tissues. Contribution to the coat was judged by the amount of agouti versus black fur. For all other tissues the contribution was determined by visual comparison of the intensity of the GPI-1^a (from the ES cells) versus the GPI-1^b (from the C57BL/6 host blastocysts) bands on a cellugel. For liver samples, six mice were analysed, and for large intestine and bone marrow, three mice were analysed. METHODS. *c-jun*^{−/−} ES cells (clones D3-2/2-3 and D3-2/4-1) were injected into 37 C57BL/6 blastocysts. From 21 mice born, 12 were chimaeras, of which 9 were males. Seven high chimaeric males were mated to C57BL/6 females. Two chimaeras generated from D3-2/2-3 and 1 from D3-2/4-1 showed transmission of the mutated allele to their progeny. Four chimaeras generated from clone D3-2/2-3 (2 females and 2 males) and two chimaeras generated from clone D3-2/4-1 (only livers were analysed) were killed and organs were analysed for GPI isozyme distribution as described²².

Rapid proteolysis of I κ B- α is necessary for activation of transcription factor NF- κ B

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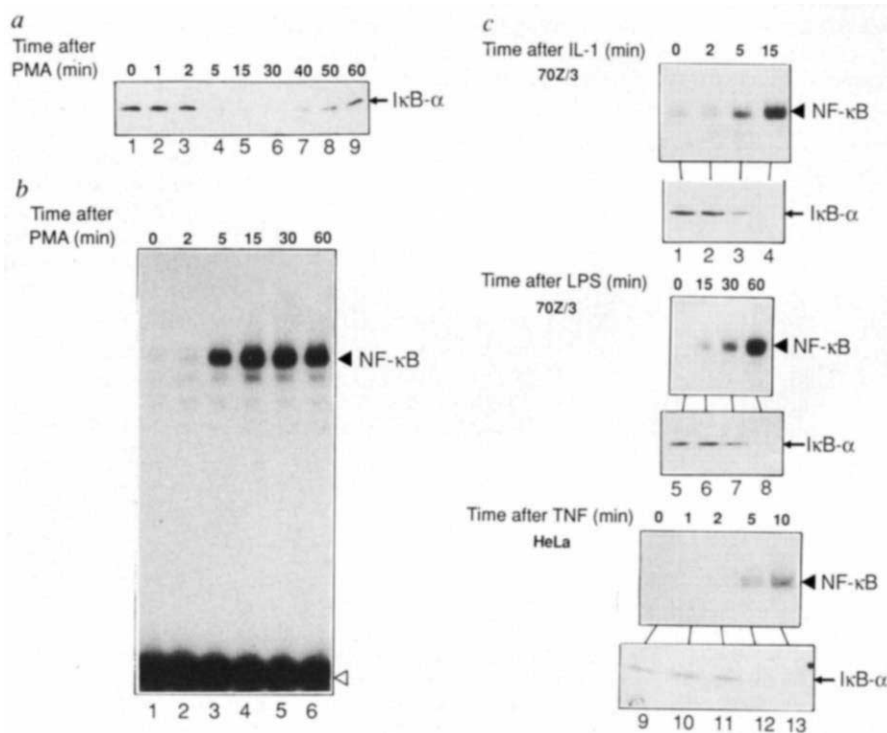
INDUCIBLE gene expression in eukaryotes is mainly controlled by the activity of transcriptional activator proteins, such as NF- κ B (refs 1–3), a factor activated upon treatment of cells with phorbol esters, lipopolysaccharide⁴, interleukin-1 and tumour necrosis factor- α ⁵. Activation of NF- κ B involves release of the inhibitory subunit I κ B from a cytoplasmic complex with the DNA-binding subunits Rel-A (formerly p65) and p50 (refs 6, 7). Cell-free experiments have suggested that protein kinase C and other kinases transfer phosphoryl groups onto I κ B causing release of I κ B and subsequent activation of NF- κ B^{8–10}. Here we report that I κ B- α

(formerly MAD-3)¹¹ is degraded in cells after stimulation with phorbol ester, interleukin-1, lipopolysaccharide and tumour necrosis factor- α , an event coincident with the appearance of active NF- κ B. Treatment of cells with various protease inhibitors or an antioxidant completely prevented the inducible decay of I κ B- α as well as the activation of NF- κ B. Our findings suggest that the activation of NF- κ B relies on an inducible degradation of I κ B- α through a cytoplasmic, chymotrypsin-like protease. In intact cells, phosphorylation of I κ B- α is apparently not sufficient for activation of NF- κ B.

We investigated the fate of I κ B- α after treatments of cells with various NF- κ B-activating stimuli. In extracts from unstimulated 70Z/3 pre-B cells, I κ B- α -specific IgG detected a single 38K band on western blots (Fig. 1a, lane 1) which was not seen with the second antibody alone (data not shown). Between 2 and 5 min after the addition of phorbol 12-myristate 13-acetate (PMA) to cell cultures, I κ B- α almost completely disappeared from cells (Fig. 1a, compare lanes 3 and 4). The specific immunoreactivity reappeared after 40 min (Fig. 1a, lane 7). The disappearance of I κ B- α coincided with the appearance of NF- κ B DNA-binding activity in total cell extracts (Fig. 1b, compare lanes 2 and 3), suggesting a causal relationship between the two events. Interleukin (IL-1 β), lipopolysaccharide (LPS) and tumour necrosis factor- α (TNF- α) all induced a decay of I κ B- α in 70Z/3 or HeLa cells (Fig. 1c), showing that distinct stimuli induced the same reaction. Although most of the I κ B- α had already decayed 5 min after stimulation with PMA, TNF- α and IL-1 β , it took more than 15 min before there was a rapid decay of the inhibitor in LPS-stimulated 70Z/3 cells. Despite these kinetic differences, the depletion of I κ B- α was in each case coincident with the

FIG. 1 The fate of I κ B- α in stimulated cells. a, The effect of PMA on I κ B- α immunoreactivity in 70Z/3 cells. Cells were treated with PMA for the indicated periods of time (lanes 2–9). Proteins in total cell extracts were separated by SDS PAGE and transferred onto a membrane filter followed by western blot analysis using anti-I κ B- α IgG and decoration with peroxidase-labelled anti-rabbit IgG. The arrow indicates the position of I κ B- α . A section of a western blot is shown. b, The effect of PMA on the DNA-binding activity of NF- κ B. Total cell extracts from control (lane 1) and PMA-treated cells (lanes 2–6) were incubated with a ³²P-labelled DNA probe encompassing the NF- κ B-binding motif from the mouse κ light chain enhancer⁴ followed by analysis of DNA-binding activities using electrophoretic mobility shift assay (EMSA). A fluorogram of a native gel is shown. c, The effect of IL-1 β , LPS and TNF on the activation of NF- κ B and stability of I κ B- α . 70Z/3 cells (upper and middle panels) or HeLa cells (lower panel) were treated with IL-1 β (lanes 2–4), LPS (lanes 6–8) or TNF- α (lanes 10–13) for the indicated periods of time followed by analysis of total cell extracts for NF- κ B activity and I κ B- α immunoreactivity. Sections of western blots and of fluorograms from native gels are shown. The position of the NF- κ B-DNA complex is shown by a filled arrowhead, the position of uncomplexed DNA probe by an open arrowhead and the position of the I κ B- α band by an arrow.

METHODS. 70Z/3 cells were cultured as described elsewhere⁴, and treated with 50 ng ml⁻¹ PMA (Sigma), 50 U ml⁻¹ IL-1 β (Boehringer Mannheim) or 15 μ g ml⁻¹ LPS (Sigma). HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum and 1% L-glutamine, and treated with 200 U ml⁻¹ recombinant human TNF- α (Genzyme). Stimulation was stopped by cooling on ice and immediate centrifugation of cells for 5 s in an Eppendorf microfuge. Cell pellets (10⁶ cells) were lysed with a high-salt extraction buffer⁷. Supernatants of lysates were used for analysis by both western blotting and EMSA, as described^{27,28}. Specificity of the protein-DNA complex was verified



by immunoreactivity with a polyclonal antibody specific for Rel-A (data not shown). Two milligrams of 6 $\times$ His-tagged, purified human I κ B- α produced in *Escherichia coli*²⁷ were coupled to 0.5 ml cyanogen bromide-activated sepharose 4CL-B (Pharmacia), according to the instruction of the manufacturer. Specific IgG in 2 ml rabbit antiserum raised against I κ B- α ²⁷ was affinity-purified²⁸ on I κ B- α -sepharose. After extensive washes with phosphate-buffered saline and 2 column vols 0.1 M glycine HCl, pH 2.7, specific antibodies were eluted with 2 vols 4 M guanidinium hydrochloride. The eluate was extensively dialysed against Tris-buffered saline and used for western blotting at a dilution of 1:100 in blocking buffer²⁸.

appearance of NF- κ B activity. In contrast to another study¹², no transient change in the electrophoretic mobility of I κ B- α was apparent after induction with the various stimuli, even when phosphatase inhibitors were included in the lysis buffer (data not shown). Moreover, we could not detect any breakdown products of I κ B- α (data not shown). Because a polyclonal antibody was used, it is unlikely that only one epitope of I κ B- α was lost or modified upon stimulation.

The protein synthesis inhibitor cycloheximide (CHX) activates NF- κ B in 70Z/3 cells⁴. Following a 1 h treatment of 70Z/3 cells with CHX, the DNA binding of NF- κ B was induced only weakly (Fig. 2a, lane 1, upper panel), and significant levels of I κ B- α were still present (lower panel). This shows that interference with the normal turnover rate of I κ B- α is not sufficient for a rapid activation of NF- κ B. When CHX-treated cells were stimulated with PMA, a rapid decay of I κ B- α and further induction of NF- κ B binding activity were observed with kinetics indistinguishable from those observed in the absence of CHX (compare Fig. 2a with Fig. 1a). But CHX prevented the reappearance of I κ B- α seen after a 40 min treatment with PMA alone (compare Fig. 2a with Fig. 1a), consistent with the finding that I κ B- α is newly synthesized under transcriptional control by NF- κ B^{12,14}. We determined a rough half-life of 138 min for I κ B- α in CHX-treated 70Z/3 cells (Fig. 2b). If protein synthesis-arrested cells were stimulated with PMA, the half-life of I κ B- α was reduced to only 1.5 min during the phase of its fastest decay, showing that PMA induced a two orders of magnitude decrease in the half-life of I κ B- α .

The functional significance of the inducible decay of I κ B- α for activation of NF- κ B was tested by exposure of cell cultures to various protease inhibitors. Six distinct serine protease inhibitors with chymotrypsin-like specificity efficiently prevent the induction of NF- κ B DNA-binding activity in response to PMA and TNF- α (Table 1). Constitutive DNA-binding activities, including Oct-1, were not affected and no significant cell death was observed by a dye-exclusion assay and phase-contrast microscopy at inhibitor concentrations preventing NF- κ B activation. Leupeptin, antipain and the trypsin inhibitor tosyl-Lys-methylester were not effective, even at high concentrations (T.M. and M.K., manuscript in preparation).

TABLE 1 The effect of various protease inhibitors on the activation of NF- κ B

Protease inhibitor	IC ₅₀ (μ M)	
	Jurkat	70Z/3
Tosyl-Phe-chloromethylketone (TPCK)	20	20
Benzylloxycarbonyl-Leu-Tyr-chloromethylketone (ZLYCK)	10	10
Tosyl-Lys-chloromethylketone (TLCK)	100	100
3,4-Dichloroisocoumarin	20	ND
N-Benzoyl-L-Tyr-ethylester (BTEE)	1,000	ND
N-Acetyl-DL-Phe- β -naphthylester (APNE)	200	200

Jurkat and 70Z/3 cell cultures were incubated with the listed compounds at various concentrations for 30 min and then stimulated by either TNF- α (Jurkat cells) or PMA (70Z/3). Total cell or nuclear extracts were prepared and analysed for NF- κ B activity using EMSA. IC₅₀, 90% inhibitory concentration; ND, not determined. Jurkat T cells were cultured as described¹⁸. All compounds (Sigma) were dissolved in DMSO and cells pretreated at various concentrations for 30 min. EMSA was done as described²⁸.

The effect of tosyl-Phe-chloromethylketone (TPCK) was investigated in more detail. Treatment of cells with 25 μ M TPCK fully inhibited the induction of NF- κ B activity (Fig. 3a, upper panels) as well as the decay of I κ B- α in response to PMA (lower panel). The half-maximal inhibitory concentration (IC₅₀) of TPCK was about 8 μ M. The protease inhibitor prevented the activation of NF- κ B by IL-1 β and LPS in 70Z/3 cells (Fig. 3b, lanes 6 and 7) and by TNF- α in various other cell lines (T.M. and M.K., manuscript in preparation). TPCK did not strongly interfere with NF- κ B activity when added after stimulation with PMA, IL-1 or LPS but seemed to arrest a further activation of the factor (Fig. 3b, lanes 10–12). It is unlikely that TPCK acted on protein kinase C (PKC) because IL-1 and TNF- α activate NF- κ B independently of PMA-inducible PKC isoenzymes^{15,16}. Furthermore, TPCK did not interfere with early signalling events of the TNF receptor¹⁷ (T.M. and M.K., manuscript in preparation). TLCK, an inhibitor of trypsin-like serine proteases, which is highly related in structure and chemical activity to TPCK,

FIG. 2 The effect of a protein synthesis inhibitor on the stability of I κ B- α and the activation of NF- κ B. a, 70Z/3 cells were treated with cycloheximide (CHX) for 1 h followed by stimulation with PMA for the indicated periods of time. Total cell extracts were assayed for NF- κ B activity by EMSA (upper panel). A section of a fluorogram from a native gel is shown with a filled arrowhead indicating the position of the NF- κ B-DNA complex. Aliquots of the cell extracts were assayed for I κ B- α by western blotting (lower panel). The position of the I κ B- α band is indicated by an arrow. b, Half-life of I κ B- α in protein synthesis-arrested cells. Cells were treated with CHX alone (left panel) or, after a 1 h treatment with CHX, induced with PMA for the indicated times (right panel). Total cell extracts of equal protein content were analysed by western blotting followed by densitometric quantitation of the 38K I κ B- α band in fluorograms. The amounts of I κ B- α detected with CHX treatment alone (left panel; open triangles) and after PMA stimulation (right panel, filled triangles) are shown on a half-logarithmic plot. Dotted lines indicate the portion of the slopes used for estimation of $t_{1/2}$ values. The dashed line in the right panel indicates the decay of I κ B- α in the absence of PMA, as determined in the left panel.

METHODS. 70Z/3 cells were treated with 25 μ g ml⁻¹ cycloheximide (Sigma). At 10 μ g ml⁻¹, protein synthesis is inhibited to 90% in 70Z/3 cells²⁹. Cell culture, extract preparation, EMSA, SDS-PAGE and western blotting were as described in the legend to Fig. 1. Densitometric scanning was done with a Howtek Scanmaster 3 and data analysed by the software Quantity One Version 2.2.

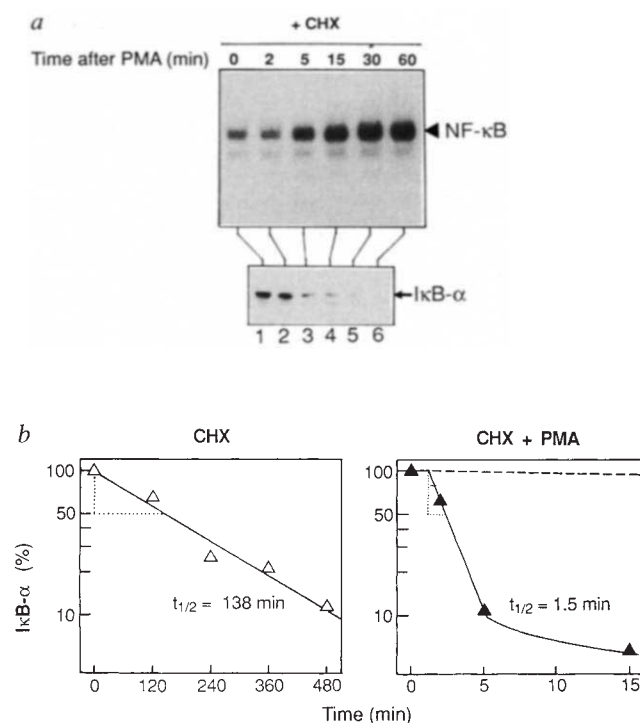


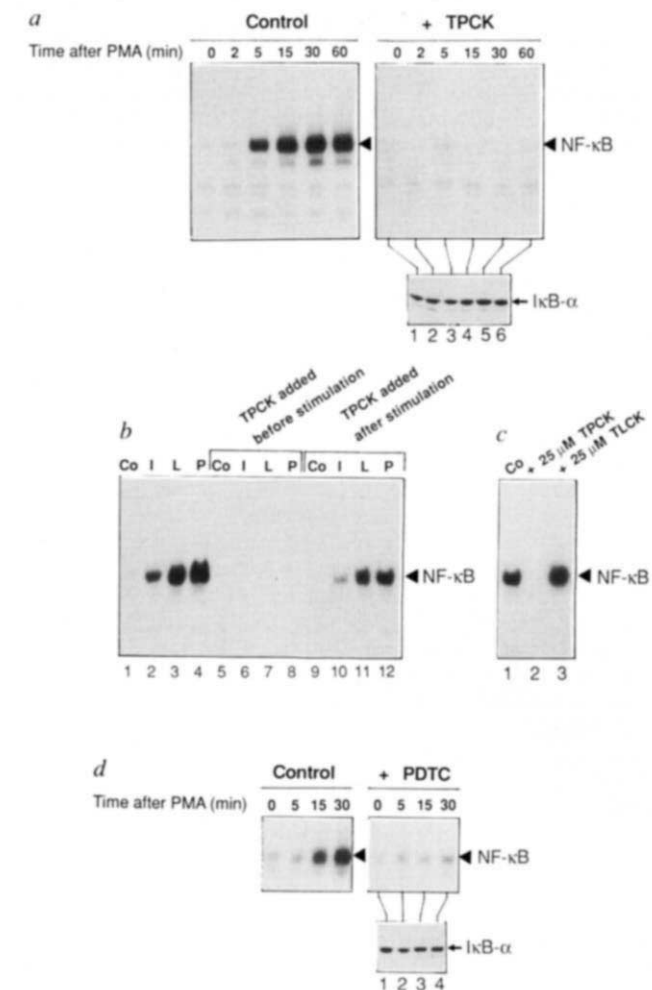
FIG. 3 The effect of various inhibitors on the activation of NF- κ B and stability of I κ B- α . **a**, The effect of TPCK. 70Z/3 cells were stimulated with PMA for the indicated times in the absence (left panel) or presence of 25 μ M TPCK (1 h pretreatment). Cell extracts were analysed for NF- κ B activity by EMSA (upper panels) and for I κ B- α levels by western blotting (lower panel). An arrow indicates the position of the 38K I κ B- α band in western blots. The faint lower band is nonspecific because its abundance was strongly reduced when affinity-purified antibody was used. The position of the NF- κ B-DNA complex in the sections of fluorograms is indicated by filled arrowheads. **b**, The effect of TPCK on the activation of NF- κ B by IL-1 β , LPS and PMA in 70Z/3 cells. Co, Control cells. Cells were treated with TPCK 10 min before stimulation (lanes 6–8) or, for 10 min, following treatments of cells for 30 min with IL-1 (L) and PMA (P), and for 60 min with LPS (L) (lanes 10–12). Total cell extracts from control (lanes 1–4) and TPCK-treated cells (lanes 5–12) were analysed for NF- κ B activity by EMSA. **c**, The effect of TLCK on the activation of NF- κ B by PMA. 70Z/3 cells were left untreated (lane 1) or treated for 10 min with either 25 μ M TPCK (lane 2) or 25 μ M TLCK (lane 3) followed by addition of PMA. Cell extracts were analysed by EMSA for NF- κ B activity. **d**, The effect of the antioxidant PDTC. Cells were treated for the indicated periods of time with PMA in the absence or presence of 100 μ M PDTC added 1 h before stimulation. Cell extracts were analysed for NF- κ B activity by EMSA (upper panels) and for I κ B- α levels by western blotting (lower panel).

METHODS. Cell culture, extract preparation, EMSA, SDS-PAGE and western blotting were as described in the legend to Fig. 1. 70Z/3 cells were pretreated with 25 μ M TPCK or TLCK (Sigma) dissolved in dimethylsulphoxide (DMSO). Control cultures received an equivalent amount of DMSO. Cell cultures were treated with the ammonium salt of PDTC (Sigma), as described in detail elsewhere³⁰.

could not prevent activation of NF- κ B at a concentration of 25 μ M (Fig. 3c, lane 3), but only at 100 μ M (Table 1). The pharmacological data strongly support the requirement of proteolytic degradation of I κ B- α for the activation of NF- κ B. The inhibitor profile suggests the involvement of a serine protease with chymotrypsin-like specificity, which we will refer to as I κ B- α protease. We cannot rule out that the protease inhibitors interfered with another proteolytic step located upstream from the I κ B- α protease. But this appears unlikely in view of the diversity of NF- κ B activators, which might have very few upstream pathways in common.

Reactive oxygen intermediates (ROIs) seem to play a role as messengers in the activation of NF- κ B by many inducing conditions^{18–20}. A potent antioxidant inhibitor of NF- κ B activation is pyrrolidinedithiocarbamate (PDTC)²⁰. No significant decay of I κ B- α or activation of NF- κ B was seen after PMA stimulation in 70Z/3 cells pre-treated with 100 μ M PDTC (Fig. 3d), suggesting the proteolysis of I κ B- α is controlled by ROIs. PDTC does not interfere with the PMA-induced membrane association and kinase activity of PKC²¹, which argues against a direct phosphorylation of I κ B- α by PKC in intact cells.

An inducible degradation of I κ B- α could have been controlled by different mechanisms. First, a covalent modification could have tagged I κ B- α for degradation by a constitutive protease. Second, the I κ B- α -protease was newly activated. The protease (or a protease inhibitor) could be a direct target for protein kinases or ROIs. Third, both tagging of the substrate as well as induction of protease activity were required. A controlled proteolytic degradation of I κ B- α is exquisitely suited for an irreversible activation of NF- κ B. The only mechanism to re-



inhibit activated NF- κ B is then through newly synthesized I κ B- α . This would explain why nuclear NF- κ B is inactivated in PMA-treated 70Z/3 cells depending on new protein synthesis²², and why I κ B- α can enter the nucleus²³ and remove NF- κ B from DNA²⁴. An important open question is whether the I κ B- α -protease can attack I κ B- α in the cytoplasmic complex with NF- κ B or only after its release from NF- κ B. A selective degradation of a subunit within a transcriptional regulator was observed with the α 2 repressor from yeast²⁵. Is phosphorylation required for the release of I κ B- α from NF- κ B? No data have yet been obtained from intact cells in strong support for an inducible phosphorylation of I κ B- α , although a constitutive phosphorylation of I κ B- α was observed (I.A. and Y.B.-N., unpublished data). Our data can only be reconciled with *in vitro* kinase data^{8–10} on the assumption that degradation is required in intact cells to eliminate phosphorylation-released I κ B- α rapidly. This would prevent I κ B- α , once it is dephosphorylated, from re-inhibiting NF- κ B. But given the strong effect of protease inhibitors, I κ B- α phosphorylation alone appears insufficient for activation of NF- κ B in intact cells. NF- κ B is critically involved in many pathological events including progression of AIDS, the acute phase response and the activation of immune and endothelial cells during toxic shock, allograft rejection, ultraviolet and radiation responses^{1,26}. We thus expect specific inhibitors of the I κ B- α protease to be effective as novel anti-inflammatory and immunosuppressive drugs. □

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Efficient catalysis of disulphide bond rearrangements by protein disulphide isomerase

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PROTEIN disulphide isomerase (PDI)^{1,2} is a highly abundant and ubiquitous eukaryotic protein that is essential for viability in yeast^{3,4}. Although PDI is thought to catalyse disulphide bond formation and isomerization during protein biosynthesis, PDI has been found previously to have only moderate effects (~25-fold) on the rate of oxidative folding of proteins *in vitro*. In addition, PDI has been implicated in several apparently unrelated cellular functions³. For example, PDI is the β -subunit of prolyl 4-hydroxylase⁵ and is part of the triglyceride transfer complex⁶. The oxidative folding of bovine pancreatic trypsin inhibitor (BPTI) is slow and inefficient *in vitro*^{7–11}. Here we report that PDI increases by a factor of 3,000–6,000 the rates of folding of kinetically trapped BPTI folding intermediates, in which native structure impedes disulphide bond formation. By contrast, PDI has only small effects on the rate of disulphide bond formation in intermediates that are oxidized readily in the absence of PDI. These results suggest that an important function of PDI is to catalyse disulphide bond formation and rearrangements within kinetically trapped, structured folding intermediates.

The best characterized disulphide folding pathway of a protein is for the *in vitro* folding of BPTI^{7–11} (Fig. 1a, b). During folding at neutral pH, BPTI accumulates rapidly as one of two intermediates. These intermediates, termed N* and N', both contain two native disulphide bonds, [5–55; 14–38] and [30–51; 14–38], respectively. About half of the molecules become trapped as N*, which is a dead-end intermediate that is stable for weeks. N' rearranges slowly (hours) to N*, and also to a third native two-disulphide intermediate ([30–51; 5–55]), termed N_{SH}^{SH}, which is oxidized readily to native BPTI (N).

Formation of the final native disulphide bond in N* and N' is hindered by native structure in these intermediates which constrains and buries the remaining free thiols^{8,9}. Recently, a naturally occurring amino-terminal pro- region (ref. 12; J. Li, S. Olson and D. A. Walz, personal communication) was shown to increase both the rate and yield of folding of BPTI¹¹. Nonetheless, even in this model of pro-BPTI, folding is slow and ~25% of the molecules accumulate as the dead-end intermediate N*.

TABLE 1 Kinetic parameters for catalysis by PDI

Transition	k_{cat} (min ⁻¹)	K_m (μM)	$k_{\text{uncat}}^\dagger$ (min ⁻¹)	Fold $\ddagger$ acceleration
N'→N	5	7	1.4×10^{-3}	3,500
N*→N	0.3	30	5×10^{-5}	6,000
proN*→proN	0.9	35	2×10^{-4}	4,500

BPTI folding experiments were done at 25 °C, 2.0 mM GSH, 0.5 mM GSSG, pH 7.3.

$k_{\text{uncat}}^\dagger$ is the rate in the absence of PDI. The values for N* and proN* are reliable only to within a factor of 2, owing to difficulties associated with measuring a rate over a period of several days under anaerobic conditions¹⁰. Michaelis–Menten parameters (K_m and k_{cat}) for the catalysis of N', N* and proN* were determined by measuring the initial rate of formation of native BPTI or pro-BPTI by HPLC. Initial velocity measurements were made at five or six substrate concentrations that ranged from 1.5 μM to 100 μM for N' and from 5 μM to 150 μM for N* and proN*. The concentration of PDI used ranged from 0.17–0.7 μM . Kinetic constants were calculated from Lineweaver–Burk plots. The initial velocity measurements were made at times in which less than one third of the starting material had rearranged. The values of k_{cat}/K_m were confirmed by measuring the rate of formation of native BPTI or Pro-BPTI for the entire time course of the reactions at initial substrate concentrations at least fivefold below K_m . Catalysis of proN' does not appear to follow Michaelis–Menten kinetics; the rate of formation of native BPTI is not constant during initial rate measurements. In addition, several well populated intermediates are present and the level of these intermediates, relative to proN', increases throughout most of the reaction. Qualitatively, however, the rate of folding of proN' in the presence of PDI is comparable to that of N' from mature BPTI.

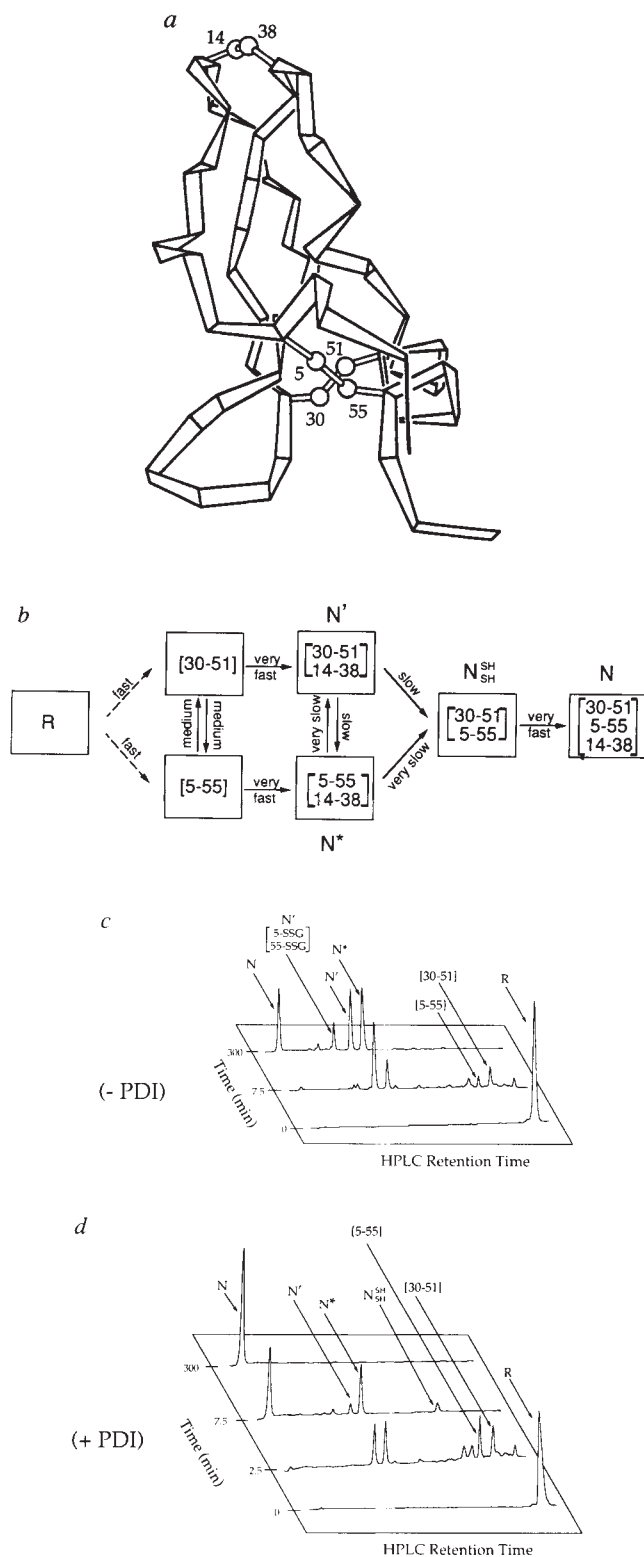
$\ddagger$ Fold acceleration is $k_{\text{cat}}/k_{\text{uncat}}^\dagger$. Under optimal redox conditions (1.0 mM GSH, 0.2 mM GSSG, pH 8.0, 25 °C) $k_{\text{uncat}}^\dagger$ for the regeneration of reduced RNase A is 0.02 min⁻¹ and k_{cat} is 0.46 min⁻¹ (ref. 16).

Earlier studies^{13,14} found that PDI increased the rate of oxidation and reduction of BPTI in the presence of dithiothreitol, but had little effect on folding when the physiological¹⁵ redox reagent glutathione was used. In those studies, however, N* was not distinguished from native BPTI (N). In addition, folding was done in the absence of a reducing agent. Subsequently it has been shown, at least for the folding of RNase A in the presence of glutathione, that catalysis of disulphide bond formation by PDI requires a redox buffer that contains reduced and oxidized components¹⁶.

We show here that PDI increases dramatically both the yield and rate of formation of native BPTI in a physiological redox buffer¹⁵ (Fig. 1c, d). The rate of formation of the two kinetically trapped native intermediates, N' and N*, is increased moderately (~3-fold) by PDI. The striking feature of the PDI-catalysed folding reaction is that the N' and N* intermediates appear to be converted readily to native BPTI (N).

Working with purified, reversibly trapped species⁹, we examined directly the effect of PDI on the folding of N' and N*. In

FIG. 1 *a*, Schematic representation of the crystal structure for BPTI^{21,22}. The residues involved in disulphide bonds are labelled. The [14–38] disulphide bond is accessible to solvent, exposing ~50% of its total surface area, whereas the [30–51] and [5–55] disulphide bonds are inaccessible, exposing 0% of their total surface area²³. *b*, Schematic representation of the kinetically preferred pathway for the folding of BPTI at pH 7.3, 25 °C^{9,10}. Intermediates are designated by the disulphide bonds they contain. Native BPTI (N) is [30–51; 5–55; 14–38]. N* denotes [5–55; 14–38]. N' denotes [30–51; 14–38]. N^{SH} denotes [30–51; 5–55]. R denotes the reduced protein. X-ray crystallographic and nuclear magnetic resonance (NMR) studies indicate that N^{SH} (ref. 24), N* (refs 25, 26), and N' (refs 9, 27; A. A. Kossiakoff, personal communication; B. A. Schulman and P.S.K., unpublished data) are folded into structures very similar to that of native BPTI. Qualitative descriptions of the relative rates¹¹ of the intramolecular transitions associated with each step are indicated. N* is a kinetically trapped intermediate that is stable for weeks under these conditions. The dotted arrows indicate that R is oxidized initially to a broad distribution of one-disulphide intermediates, which then rearrange rapidly to [30–51] and [5–55]. *c*, *d*, Time course of folding of reduced BPTI in the absence (*c*) or presence (*d*) of 1.5 μ M PDI. A folding reaction was initiated by the addition of redox buffer to reduced BPTI (10 μ M). At the indicated times, a portion of the sample was quenched with acid, and analysed by high-performance liquid chromatography (HPLC). N'[5–SSG; 55–SSG] denotes the double mixed-disulphide derivative of N', in which Cys 5 and Cys 55 are each disulphide-bonded to glutathione. **METHODS.** The folding reactions of BPTI in the presence or absence of PDI are monitored as described^{9–11}. Briefly: (1) folding is initiated by the addition of degassed pH 7.3 folding buffer (150 mM NaCl, 100 mM sodium phosphate, 1 mM EDTA, pH 7.3) containing 2.0 mM GSH, 0.5 mM GSSG, to reduced BPTI; (2) at various times, a portion of the folding reaction is quenched with 0.1 volumes of 3M HCl and; (3) the spectrum of intermediates present at each time is determined by HPLC in acidic conditions (pH 2). The disulphide linkages of the observed intermediates have been determined previously⁹. All folding experiments were done in a circulating water bath at 25 °C. The redox buffer was chosen to mimic the redox conditions of the endoplasmic reticulum¹⁵. Bovine PDI was purchased from Pierce and was ~90% pure as judged by reverse-phase HPLC and SDS–PAGE. As noted previously²⁸, two forms of PDI, thought to be monomeric and dimeric species, are resolved by HPLC gel filtration (Toso Haas G2000SW $\times$ 1). Using this assay, the preparation of PDI was judged to be ~85% monomeric.



each case, the rate of disulphide bond formation is increased dramatically by PDI (Fig. 2). These enzyme-catalysed reactions appear to follow Michaelis–Menten kinetics (Table 1). Compared to the uncatalysed rates, the k_{cat} values for the PDI-catalysed folding of N' and N* are 3,500- and 6,000-fold faster, respectively (Table 1). In contrast, PDI increases only moderately the rate of disulphide bond formation in N^{SH} (Fig. 2c), an intermediate that, unlike N' and N*, forms native BPTI readily even in the absence of a catalyst. Similarly, PDI affects only

moderately the rate of disulphide bond formation in the fully reduced protein, R, and in the single-disulphide intermediates [30–51] and [5–55] (Fig. 1c, d). Thus, the large effects of PDI are specific to the kinetically trapped intermediates.

Productive folding of pro-BPTI¹¹ also proceeds through the [30–51; 14–38] intermediate (termed proN'). A significant portion of pro-BPTI also becomes trapped as the dead-end species [5–55; 14–38] (termed proN*). As with N* and N', PDI increases dramatically the rate of productive disulphide bond

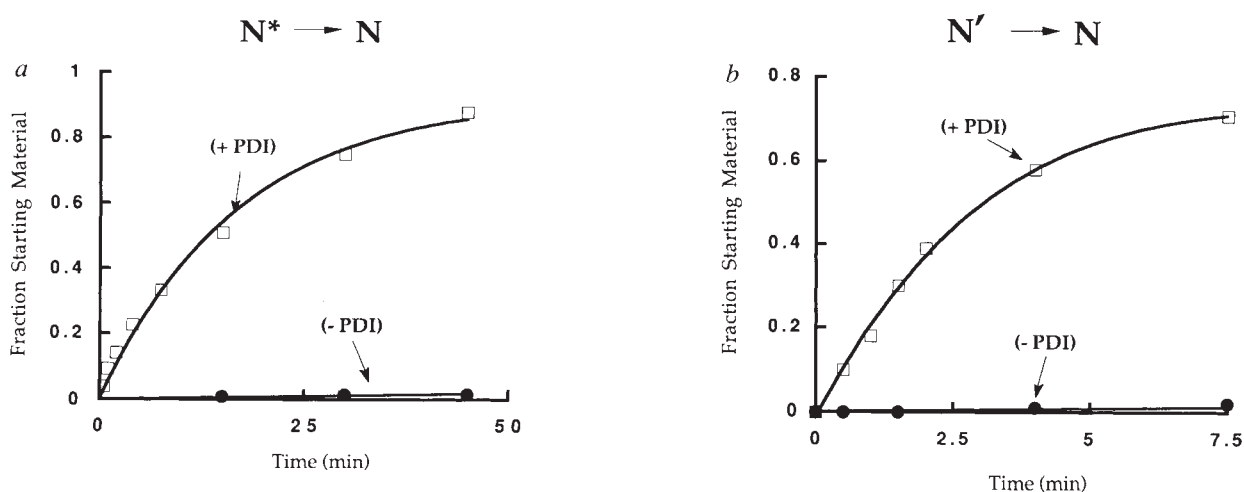


FIG. 2 Effects of PDI on the productive folding of each of the native two-disulphide intermediates (N^* , N' and N_{SH}^{SH}). The concentration of BPTI in these studies is $35\ \mu\text{M}$ and the concentration of PDI is $7\ \mu\text{M}$. a, Folding of N^* . b, Folding of N' . In the last time point shown (7.5 min), ~20% of the starting material has accumulated as the N^* intermediate. c, Folding of N_{SH}^{SH} . Because the rate-limiting step in the oxidation of N_{SH}^{SH} is the intermolecular reaction of a protein thiol with GSSG, it is possible that PDI enhances substantially the rate of the intramolecular steps associated with the oxidation of N_{SH}^{SH} while having little effect on the overall rate of disulphide bond formation.

METHODS. Reversibly trapped intermediates were produced as described⁹. Folding was initiated by addition of pH 7.3 folding buffer containing 2.0 mM GSH, 0.5 mM GSSG to the purified intermediate in the presence or absence of PDI. At various times, an aliquot of the folding reaction was quenched with acid and analysed by HPLC. The fraction of native BPTI (N) present in the HPLC chromatograms was determined by UV absorbance (at 229 nm), assuming that all of the intermediates have identical extinction coefficients. The initial rate of formation of N during the PDI-catalysed folding of N' is somewhat smaller than expected on the basis of the kinetic parameters (Table 1). This is due in part to the transient accumulation of the N_{SH}^{SH} intermediate at high PDI concentrations (Fig. 3).

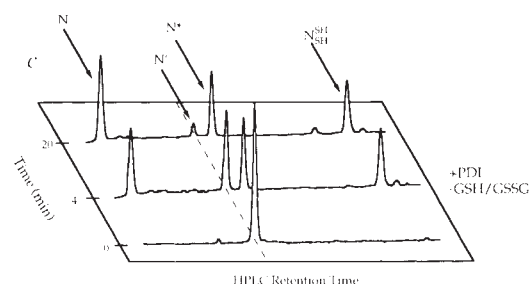
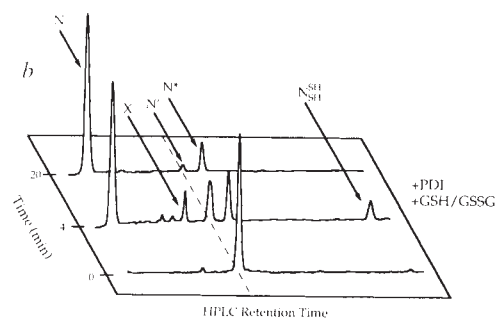
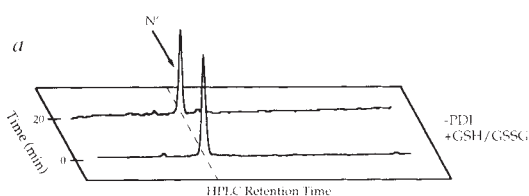
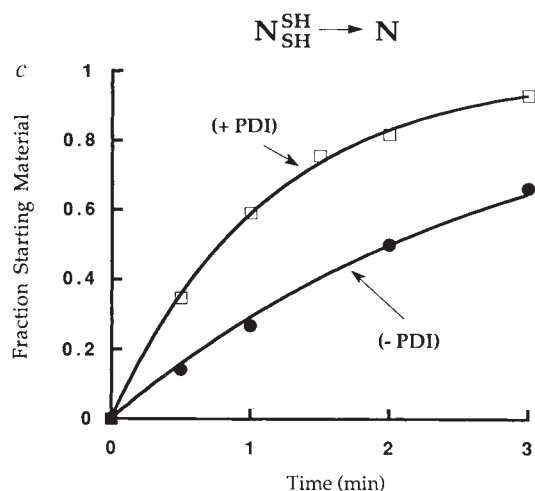


FIG. 3 Catalysis by PDI of the intramolecular rearrangement of N' . a, Stability of N' in pH 7.3 folding buffer containing 2.0 mM GSH, 0.5 mM GSSG. b, Rearrangement of N' in the presence of PDI and pH 7.3 folding buffer containing 2.0 mM GSH, 0.5 mM GSSG. The peak denoted by X is tentatively assigned as the double mixed-disulphide derivative of N' ($N'[5\text{-SSG}; 55\text{-SSG}]$) on the basis of its HPLC elution time. c, Rearrangement of N' in the presence of PDI and pH 7.3 folding buffer lacking GSH and GSSG. Despite the absence of GSSG, a significant fraction of the starting material forms native BPTI (N). The disulphide bonds in PDI and/or residual oxygen in the buffer could be acting as the oxidizing agent.

METHODS. Folding experiments are described in Fig. 1c, d. The concentration of N' was $35\ \mu\text{M}$ and the concentration of PDI was $3.5\ \mu\text{M}$.

formation in both proN* and proN' (Table 1). This suggests that PDI is primarily responsible for the rapid rate of BPTI folding *in vivo*.

The conclusion that PDI principally accelerates the folding of kinetically trapped intermediates, such as N' and N*, provides an explanation for why the effects of PDI on the folding of BPTI are much greater than on RNase A. The folding of reduced RNase A is relatively rapid even in the absence of PDI¹⁶ and therefore does not appear to be retarded substantially by the accumulation of structured intermediates. By contrast, formation of the native state of BPTI is hindered by the accumulation of highly structured intermediates. Thus, although the uncatalysed rate for folding of BPTI is substantially lower than for RNase A, the PDI-catalysed rates for folding are similar in the two proteins (see Table 1 legend).

PDI could help formation of the final disulphide bond in the kinetically trapped intermediates, N' and N*, either by accelerating the intramolecular rearrangement of these intermediates (to N^{SH}_{SH}) or by catalysing the direct oxidation of a third native disulphide bond. In the case of N', we find that the other native two-disulphide species (N^{SH}_{SH} and N*) accumulate rapidly in the presence of PDI (Fig. 3b). Moreover, PDI catalyses the rearrangement of N' (to N^{SH}_{SH} and N*) in the absence of redox reagents (Fig. 3c). These observations demonstrate that PDI acts largely by increasing the rate of intramolecular rearrangement steps, although it is possible that PDI also accelerates direct oxidation (see also ref. 13).

The mechanism by which PDI catalyses disulphide bond rearrangements in structured intermediates is not known. It is known, however, that addition of high concentrations of denaturant (6 M urea) accelerates the rate of rearrangement of the N' (ref. 9) and N* (ref. 8) intermediates, suggesting that the rearrangement of these species requires substantial loss of structure¹⁰. In addition, PDI has been observed to promote the reductive unfolding of structured intermediates of BPTI¹³ and retinol-binding protein¹⁷. Finally, PDI is able to interact with a wide variety of unstructured peptides^{18,19} in a manner similar to the Hsp70 family of molecular chaperones²⁰. These considerations raise the interesting possibility that PDI functions in part by promoting both local unfolding and disulphide bond rearrangements in structured intermediates. □

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ACKNOWLEDGEMENTS. We thank A. A. Kossiakoff and D. A. Walz for communicating unpublished results and H. F. Gilbert for discussion. P.S.K. is a Pew Scholar in the Biomedical Sciences. This research was supported by a grant from the NIH.

ADDENDUM

The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation

Marc G. Weisskopf, Robert A. Zalutsky & Roger A. Nicoll

Nature **362**, 423–427 (1993)

SINCE publication of our article, it has become apparent that further reference to some topics covered in this paper is warranted. First, reference should have been given to research in invertebrates establishing functional roles for colocalization of neuropeptides with other neurotransmitters. This area of research is discussed thoroughly in a recent review¹. Second, although not the major focus of the article, our finding that the opioid receptor antagonist naloxone has no effect on hippocampal mossy fibre LTP conflicts with another report². Apart from the different species, experimental conditions and *in vitro* slice preparation used in our study, rather than an *in vivo* anaesthetized preparation², we have no explanation for the difference in results. □

1. Kupfermann, I. *Physiol. Rev.* **71**, 683–732 (1991).

2. Derrick, B. E., Weinberger, S. B. & Martinez, J. L. *Brain Res. Bull.* **27**, 219–223 (1991).

CORRECTION

Pulsed high-energy γ -rays from the radio pulsar PSR1706–44

D. J. Thompson, Z. Arzoumanian, D. L. Bertsch, K. T. S. Brazier, N. D'Amico, C. E. Fichtel, J. M. Fierro, R. C. Hartman, S. D. Hunter, S. Johnston, G. Kanbach, V. M. Kaspi, D. A. Kniffen, Y. C. Lin, A. G. Lyne, R. N. Manchester, J. R. Mattox, H. A. Mayer-Hasselwander, P. F. Michelson, C. v. Montigny, H. I. Nel, D. Nice, P. L. Nolan, K. Pinkau, H. Rothermel, E. J. Schneid, M. Sommer, P. Sreekumar & J. H. Taylor

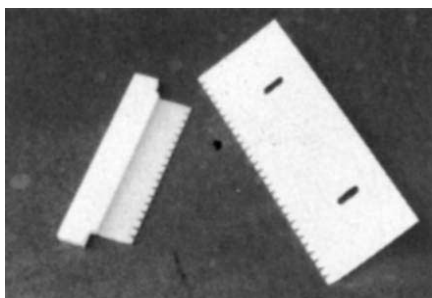
Nature **359**, 615–616 (1992)

IN Fig. 2 of this letter, the y-axis should be labelled as Photons (cm² s MeV)^{−1} and not Photons (cm² s GeV)^{−1} as published. □

PCR primer

This week's sampler includes a new kit for the rapid purification of polymerase chain reaction (PCR) products, a new matrix for isolating PCR-quality DNA and a kit for PCR amplification of the 5' ends of cDNAs.

THE trapezoidal tooth design of the TaperTooth comb from Gensura Laboratories creates 3- or 4-mm deep wells in standard 4- or 5-mm thick analytical agarose gels (*Reader Service No. 100*). Gensura says that **triangular-shaped agarose well dividers** reach their apex at the gel surface, reducing the effective lane spacing to less than 1 mm. This design increases the lane number by



Gensura's new gel comb design — for increased capacity, closer lane spacing and easier removal.

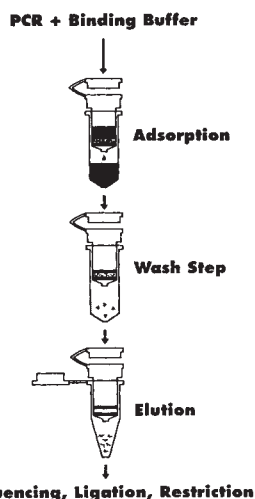
20–30 per cent over conventional combs and facilitates accurate lane-to-lane mobility comparisons — making them suitable for screening multiple PCR samples. Gensura says that the tooth sides pull cleanly away from agarose as the comb is lifted. The teeth are made of 1-mm thick Delrin; all combs are custom manufactured to individual specifications, with either a thickened integral Delrin spine for slotted gel trays or with holes to fit any bridge-and-screw style apparatus. They are supplied in a choice of 3-mm or 4-mm tooth depth and 3-mm or 4.5-mm lane spacing — compatible with loading by multichannel pipettors.

SMACK I is the new **size marker and control kit** from Advanced Biotechnologies (*Reader Service No. 101*). A dual-purpose kit for use in PCR, which acts as a combined positive control and DNA size ladder, yielding DNA fragments of the following sizes: 1,770, 960, 692, 435 and 331 base pairs. The conditions have been optimized to give even band intensity for each amplified fragment using a robust temperature cycle profile. In addition, the company offers a new and improved single-step method for the isolation of total RNA from tissues, cells, bacteria, plants, yeast and biological fluids. The procedure is said to take less than an hour and presents the RNA undegraded, and free of protein and DNA contamination. The RNA can subsequently be used for northern analysis, dot-blot hybridization, poly A⁺ selection, *in vitro* translation, RNase protection assays, molecular cloning and for com-

pared RT/primer extension procedures without additional treatment with DNase.

Purification

The new QIAquick-spin PCR purification kit from Qiagen is designed to provide a fast and efficient way of purifying PCR products (*Reader Service No. 102*). Both single- and



QIAquick-spin purification kit procedure.

double-stranded products are purified in minutes on the special QIA-quick-spin column, says Qiagen: there is no need for extra mineral oil or paraffin removal steps. The kit takes advantage of the selective properties of a silica membrane and the speed of micro-centrifugation to provide recoveries of greater than 90 per cent over a wide range of

Recovery of different PCR fragments after purification over QIAquick-spin columns

PCR fragment size (base pairs)	Recovery after QIAquick-spin column purification (per cent)
100	90
500	95
1,000	95
2,000	93
3,000	91

product sizes — 100 base pairs (bp) to greater than 3 kb. Primers and primer-dimers are removed from products as small as 100 bp. Qiagen says that the kit yields product pure enough for manual or automated sequencing. Two sizes of the kit are available for 50 and 250 preparations. All columns and reagents for PCR product purification are provided in the kit.

Isolate template DNA and visualize PCR product all in the same day with Bio-Rad's new InstaGene **matrix for PCR-quality DNA** (*Reader Service No. 103*). Use of the matrix is designed to eliminate the time-consuming and often labour-intensive deproteinization, organic extraction, dialysis and alcohol precipitation steps associated with traditional genomic DNA purification protocols. The procedure is designed to be fast and simple: place cells in a microfuge tube, add InstaGene matrix, boil and spin. PCR-ready DNA is removed from the supernatant directly to PCR reactions. Bio-Rad says the InstaGene matrix adsorbs cell lysis products that interfere with the PCR amplification process. Single-tube isolation procedures and the reduced number of manipulations with this kit should help to decrease inadvertent cross-contamination. The matrix is intended for large- or small-scale PCR screening procedures and has been performance tested using a functional PCR assay to ensure amplification-quality DNA.



Matrix for PCR-quality DNA.

Dynabeads mRNA DIRECT kit is designed for the **isolation of purified, intact mRNA directly from crude samples** (*Reader Service No. 104*). Included in the kit from Dynal are reusable superparamagnetic Dynabeads Oligo (dT)₂₅, which eliminate the need for spin columns, filtration, organic reagents and ethanol precipitation. Only a minute amount of starting material is required and the purification is performed in a single tube. Dynal says that a strong RNase-inhibiting agent, together with stringent hybridization and washing conditions, ensure the isolation of intact, high-purity mRNA. The purified mRNA can be used in RT-PCR, solid-phase cDNA library, S1 nuclease analysis, ribonuclease protection, primer extension, dot and slot hybridization, *in vitro* translation experiments, subtractive hybridization and northern blotting. Dynal says that the isolated mRNA is suitable for gene cloning and gene expression analysis.

Until now, molecular studies of archived tissue required lengthy organic extraction and digestion. Now with the new Exwax paraffin-embedded **DNA extraction kit** from Alpha Laboratories, researchers can start with a tissue block in the morning and

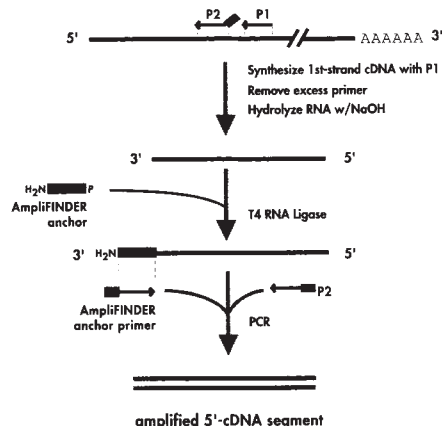
PRODUCT REVIEW

perform PCR that same night (*Reader Service No. 105*). The company says that archived blocks can be extracted in seven hours. It is suitable for obtaining template DNA for PCR products of 400 bases or more. There is no organic disposal.

Purifying DNA from chorionic villi samples (CVS) and direct amniotic fluid can be a tedious manual procedure. However, with the Genepure 341 **nucleic acid purification system** from Applied Biosystems, this procedure is now fully automated (*Reader Service No. 106*). The system is designed to provide improved reliability and consistency for small-sample extraction, while reducing the risk of mixing up samples and exposure to toxic substances. Genepure offers two automated chemistry options, standard and fast cycle, for purifying small quantities of DNA that is suitable for PCR and Southern blot analysis. ABI says that the stringency of the standard phenol/chloroform method is recommended for all samples and sizes. It yields purified DNA that is suitable for any type of analysis. Because of their small size and relatively low protein content, CVS and amniotic samples are also good candidates for the fast cycle chemistry, a rapid, nonorganic alternative.

■ Kits

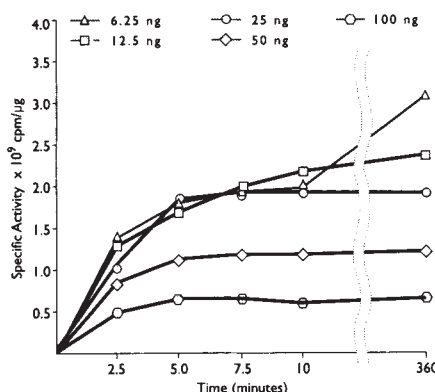
The new 5'-AmpliFINDER RACE kit from Clontech is designed to allow efficient **PCR amplification of the 5' ends of cDNAs** (*Reader Service No. 107*). Obtaining the 5'



With Clontech's new kit, PCR amplification of the 5' ends of cDNAs is designed to be a snap.

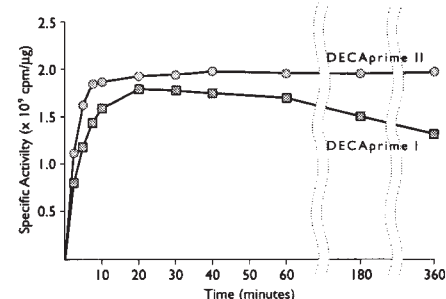
end coding region of a gene is critical for gene expression and structural studies. However, the 5' sequences can be difficult to obtain, even after weeks or months of screening cDNA libraries. With the 5'-AmpliFINDER method, cDNA is first synthesized from poly A⁺ RNA using a gene-specific primer. An AmpliFINDER anchor oligonucleotide is then ligated to the cDNA — the anchor is modified with an amino group to prevent self-ligation, and contains an *EcoR*I site for use in cloning the amplified fragments. The anchor-ligated cDNA is then amplified by PCR using the AmpliFINDER anchor primer and a nested gene-specific primer. Clontech says that PCR products can be easily cloned for obtaining the sequence of the 5' end of the cDNA. This method provides an advantage over the original 5' RACE method, which requires homopolymeric tailing, an often inefficient and problematic step.

Ambion announces the availability of its new **DECAPrime II DNA labelling kit** for random primer or oligo-primed synthesis of high-specific activity probes (*Reader Ser-*



Specific activity of [α -³²P] dATP (50 μ Cl, 3,000 Ci mmol⁻¹) in a DECAPrime II reaction using the indicated amounts of the 3 kb control template DNA in a 25 μ l reaction volume incubated at 37 °C. Aliquots were removed at the indicated times to access total and acid precipitable counts.

vice No. 108). The kit is said to offer significant improvements in reaction time, convenience and, most importantly, in synthesis of higher specific activity probes when compared to the original DECAPrime kit and other conventional random priming reactions. The use of exonuclease-free Klenow polymerase and optimized reaction buffers allows the synthesis of probes with greater than 10⁹ c.p.m. per microgram with 5 min reaction times (see fig., below left) and results in probes with higher specific activity (see below). The figure, below left, illustrates the result that the smaller the amount of starting template DNA, the higher the specific activity of the probe. This effect is enhanced using exo-minus Klenow polymerase since template is not degraded during reactions, containing small amounts of tem-



Specific activity of a DECAPrime versus DECAPrime II probe synthesis reaction. The incorporation of [α -³²P]dATP (50 μ Cl, 3,000 Ci mmol⁻¹) using 25 ng of a 3 kb template DNA at 37 °C for the indicated times was used to determine the specific activity of the probe synthesized.

plate, which are left to go to completion. The elimination of exonuclease activity is useful in situations where DNA quantitation is in doubt and the amount of DNA being labelled is not precisely known or less than 25 ng. Ambion says that for small samples of DNA template, or when the DNA sample is 'dirty' and may label at a lower rate, the incubation time may be extended to maximize both the yield and the specific activity of the probe without the need to determine empirically the optimal reaction time.

Perkin-Elmer's GeneAmplicon HLA DRB biotinylated probe sets I and II are designed to provide a sensitive, reproducible **PCR-based analysis of human leukocyte antigen (HLA) Class II loci** used in tissue-typing research, as well as in parentage testing, forensic analysis, and human cell-line tracking (*Reader Service No. 109*). These probes enable users to distinguish 60 DRB1 alleles in every possible heterozygous combination. With the GeneAmplicon HLA DRB probe set I, samples need only DNA, not Class II positive cells. The typing is reproducible and shows differences in alleles and hybridization patterns for every type. Complete protocols are included in the set for conducting PCR-based HLA typing. In addition, the reagents are synthetic, therefore

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eliminating the inconsistency associated with reliance on antisera. The company's corresponding GeneAmp primer HLA DRB Allele-specific Primer Set is designed to make detection of HLA Class II loci easy, reliable and sensitive when used with the probe sets. They can also be used for the analysis of HLA polymorphisms or to study the relationship between HLA variants and susceptibility to disease.

■ Thermal cyclers

The UNO-Thermoblock from Biometra is a compact **thermal cycler** designed for research, routine and diagnostic applications in molecular biology (*Reader Service No. 110*). A smaller version of the TRIO-Thermoblock, the UNO-Block is available in two versions: the UNO-Thermoblock 96



Two versions of the UNO-Thermoblock are available for 40 or 96 tubes, and plates.

for 96 tubes (0.2 ml) or microtitre plates, and the UNO-Thermoblock 40 for 40 tubes (0.5 ml). A simple modular exchange converts the UNO 96 into the UNO 40, or the reverse. Fast and precise temperature profiles can be driven in the temperature range from +4 °C up to 100 °C. The UNO-Block comes with TRIO software and additional options like the 'hold' function for the 'hot start' reaction. Heating and cooling rates (1.5 °C s⁻¹ and 1.3 °C s⁻¹, respectively) can be reduced via ramping functions down to 0.01 °C.

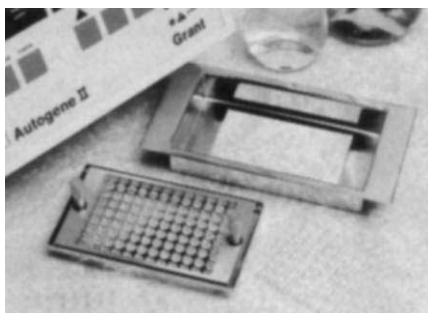
Integra Biosciences is now marketing the Cyclone fully automated **thermal cycler** system (*Reader Service No. 111*). The Cyclone features Peltier element technology for smooth heat transfer (with a stated accuracy of 0.5 °C between 15 °C and 100 °C) and rapid temperature changes without the need for viscous fluids. The accompanying software offers a choice of 50 programmes. A printer can be connected, enabling all data to be printed for subsequent evaluation. Three interchangeable incubation blocks for use with 0.5 and 1.5 ml microcentrifuge tubes, as well as microtitre plates, are available.

The GeneAmp 9600 **thermal cycler** manufactured by Perkin-Elmer and supplied outside the United States by Roche Diagnostic Systems to diagnostic laboratories, paternity and veterinary service licensees of F. Hoffman-La Roche, provides automation of the PCR process and is recommended for the Amplicor range of diagnostic kits (*Reader*

Service No. 112). The thermal cycler's 96-well format makes it compatible with other standard 96-well microplate laboratory equipment and procedures. Self-contained heating, cooling and control systems within the GeneAmp 9600 thermal cycler allow regulation of sample temperature over the range of 4 °C to 99.9 °C with a stated resolution of 0.1 °C. Specially designed thin-walled MicroAmp reaction tubes are designed to provide precise sample temperature control and rapid cycling. Up to 150 customized run programmes can be stored.

Using a new method of DNA *in situ* amplification, two independent research teams at the National Institutes of Health and the University of Minnesota have demonstrated that HIV infection is active and progressive in specific lymphoid tissues throughout the disease. The MJ Peltier-driven **thermal cycler**, the PTC-100-MS, available from Genetic Research Instrumentation, has been developed specifically for this kind of work and has a block designed to accept up to 12 standard glass microscope slides (*Reader Service No. 113*). Protocols and technical data are available, together with copies of papers published recently using this instrument. The microprocessor-controlled PTC-100-MS has a stated accuracy of better than 0.5 °C per slide and is supplied with protocols developed specifically for *in situ* amplification.

The Autogene II **temperature-cycling water bath** from Grant Instruments now accepts microplates (*Reader Service No. 114*). The company says that the tempera-



Autogene II accepts microplates.

ture uniformity and accuracy of Autogene II ensures that all sample wells undergo the same time/temperature profile. In addition, the microtitre plate is made of a heat-resistant plastic for high-temperature operation. Other design features include a specially-designed holder to make handling easy and two alternative sealing methods to ensure integrity of the sample. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. PCR is covered by patents owned by Hoffman-La Roche, Inc.

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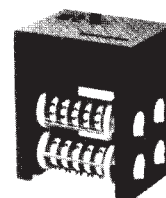
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